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The Journal of Experimental Biology

EDITED BY

V. B. WIGGLESWORTH and J. A. RAMSAY

Contents

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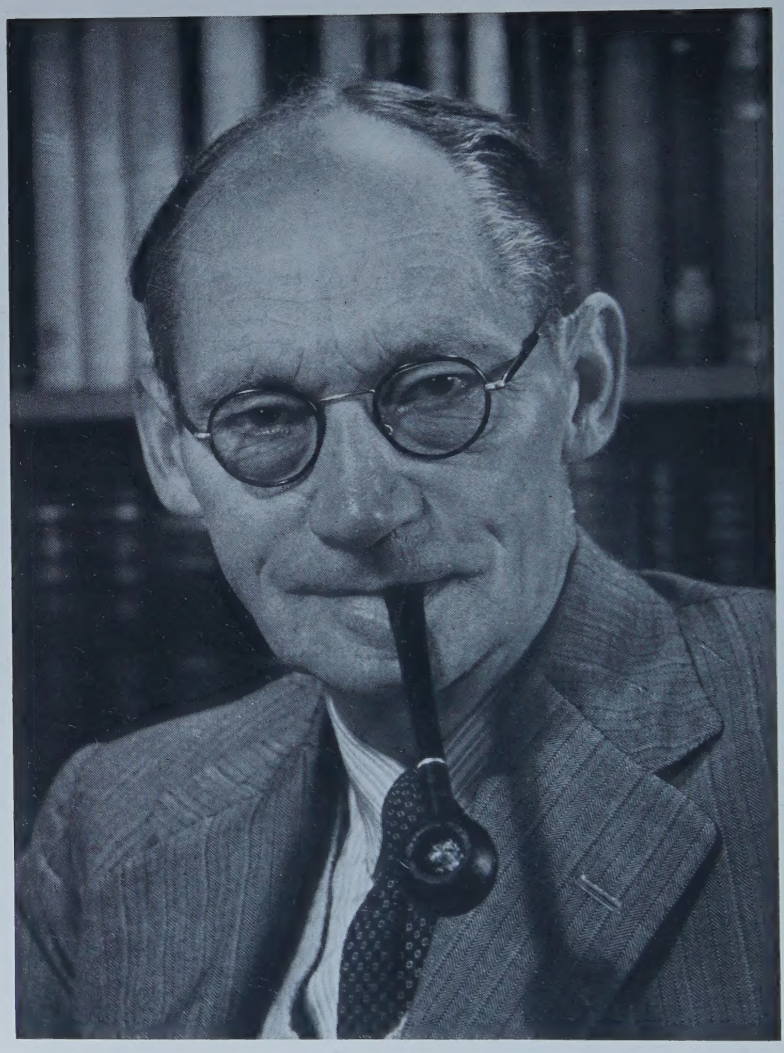

Photo by Barrington Brown

James Gray

THE JOURNAL AND ITS EDITORS

This number marks an important event in the history of this journal. Sir James Gray, who as senior editor has directed editorial policy almost since its foundation, has asked to be allowed to lay down his burden. In his place the Company of Biologists have appointed Professor V. B. Wigglesworth. Their choice will be applauded by biologists generally. Moreover, he will continue to have the able help of Dr J. A. Ramsay, and the change of editorship carries with it no change in general editorial policy.

Readers and contributors will wish the new editor all success. He inherits a journal which in the course of some thirty years has come to hold a very high place amongst the World's scientific publications. In reaching that position it has materially influenced the science of biology during a period of very active growth. This is the work of the retiring editor.

The journal began just after the First World War. The previous decade had seen the end of that great phase of classificatory morphology which had dominated zoology, and to a less extent botany, during the latter half of the nineteenth century. As an inspiration for research it had, for the time, become sterile, particularly in zoology. The year 1918 was a time of breaking traditions. In natural science it put a period to a phase which began in the seventeenth century with the foundation of the Royal Society. The young zoologists and botanists of 1918 were not content with the old morphology. They turned their attention to the living organism, to the relation of structure to function, to the physico-chemical basis of living processes and to the relation of living things to their environment. In this they were joined by the physiologists who, largely under the powerful influence of Bayliss's *General Physiology* had come to realize that their subject extended far beyond man and the familiar laboratory vertebrates. The common feature in the heterogeneous assembly of their researches was interest in the living organism and in the use of experiment as well as observation to reach conclusions.

In this way 'experimental biology' came into existence. In spite of occasional difficulties its original wide range is still maintained by the Society for Experimental Biology now nearing its hundredth meeting. Those engaged in such researches required a journal of new scope for their publications. Very bravely in October 1923 a group of biologists launched the first number of the *British Journal of Experimental Biology* with an editorial board under Professor F. A. E. Crewe as managing editor. But this new journal was almost at once surrounded with difficulties. It soon became apparent that the journal would receive its greatest support from the 'animal' side. Practical difficulties attendant upon any attempt to disregard the traditional divisions of biological organization proved to be rather more than a youthful idealism could surmount. The needs of the experimental zoologist and those of the experimental botanist were not in practice the same. Plant physiology and the study of the physical environment of plants has always been very

properly included in botany. And though the experimental botanist had difficulties about publication—as the recently started *Journal of Experimental Botany* bears witness—it was at least possible for the plant physiologist to bring much of his work before the public he desired.

Similarly, it proved undesirable for the new journal to overlap too completely with others already existing, such as those catering for genetics and traditional physiology. Whether we like it or not, in the divisions of scientific publications there is a powerful historical element which cannot be disregarded. Just as the present divisions of the sciences themselves are profoundly influenced by the accident of seventeenth- and eighteenth-century benefactions to our universities and medical schools, so must the field of any new journal be influenced by the character of journals already established. But the word 'biology' in our title reminds us of the grand idea that underlay the foundation of the journal. And if the 'biology' has in practice become essentially restricted to that of animals, it still reminds us that these are to be considered only as part of the whole array of living things. May no future pedant change our title.

Whilst the experimental botanists were not without their difficulties, those of the experimental zoologists were more serious. To-day we so naturally take for granted a broad view of the content of zoology that it is not easy to recapture the old atmosphere of dissension and bitterness about the scope of the science. With certain honourable exceptions the older generation of zoologists of the early twenties looked askance at the new venture. For many years 'experimental biology' was treated by many as a bastard science outside the pale of pure evolutionary morphology whose boundaries—curiously—were considered to be coterminous with those of zoology itself. There was at first no journal to receive and welcome the work of the experimental zoologist.

But as might be expected, the primary difficulty of the *British Journal of Experimental Biology* was financial. The first number of the new journal announced the intention to form a 'British Association of Experimental Biologists: to promote intercourse between experimental biologists within the United Kingdom and to encourage facilities for the publication of experimental work'. This was the embryo of the Society for Experimental Biology which came into existence in December 1923. From its first meeting the Society was a success. It had the enthusiasm of youth: nearly everyone concerned in these events was in the twenties or early thirties. In these more highly organized days, youth is not so well served with opportunity to mould things to its desire. But though young people are active and enthusiastic they are commonly penurious, and when a motion was put that the journal be financed by every member subscribing to it, the motion was rejected. And though the journal was acclaimed the official medium of publication of the Society, the problem of financing it was left unsolved.

Another difficulty that beset the *British Journal of Experimental Biology* was this: there already existed another journal in this new field. There was a small but active group of experimental zoologists at Cambridge. Gray, our retiring editor, had persuaded the Cambridge Philosophical Society to divide their *Proceedings* into

Physical and Biological Sections. They appointed him editor of their *Biological Proceedings* and the very success of this venture cut off a not unimportant section of experimental zoologists from the *British Journal of Experimental Biology*. With all the uncertainties ahead there might be room for one journal, there was certainly none for two.

Something had to be done and in the negotiations which ensued a leading part was played by the late Dr G. P. Bidder whose wise advise and timely help have done so much for British biology during the last fifty years. A limited liability company, the Company of Biologists, was formed with the object of owning and publishing scientific journals. A number of biologists, the majority being members of the Society for Experimental Biology, were invited to become shareholders and with the capital thus raised the *British Journal of Experimental Biology* was taken over from the firm which was then publishing it. As time went on more shares were bought, a number by the Society for Experimental Biology itself, and in this way the journal was given support in its early years. Needless to say the shareholders did not expect or receive any dividend but their subscriptions have recently been repaid and the Company has reformed as a company limited by guarantee, without share capital, a form of association which has enabled it to become recognised as a charity.

The creation of the Company introduced an important new principle: the separation of the financial from the editorial side of publication. By its articles the Board of the Company cannot interfere with the policy of its editors. One of its earliest acts brought in another principle of equal importance. The original *British Journal of Experimental Biology* was edited by a Board. In well-defined branches of science with well-established standards, this method of editing has sometimes worked well. But the new journal was faced with problems of every kind including the setting of a high and consistent standard in new parts of biology. For such a journal that latter-day nautical curiosity, the 'steering committee', was a wholly unsuitable substitute for an editor; provided an editor with really wide knowledge and with sagacity could be found. After a few rapid intermediate arrangements, the Board appointed Gray as editor of the journal, the journal was transferred to its present printers, the Cambridge University Press, and its title was changed to its present one by the omission of the word 'British'.

With Gray's appointment to the *British Journal of Experimental Biology* he ceased to edit the *Biological Proceedings of the Cambridge Philosophical Society*. This was transformed into a journal of a different sort, the now well-known *Biological Reviews*.

Since 1926 publishing of all kinds has gone through many vicissitudes. But from the smallest of beginnings the retiring editor has brought the *Journal of Experimental Biology* to a state in which it is recognized as one of the leading biological journals, a journal which combines its wide field of interest with a high and consistent standard. That is a lasting gain. For the state of Natural Science in any age is more surely reflected in the quality of its scientific publications than in the magnificence of its institutions and the expense of its equipment. C. F. A. P.

ON THE REACTION TO LIGHT OF *MYXINE GLUTINOSA* L.

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(*Received* 20 *February* 1954)

That the effectively eyeless hag, *Myxine glutinosa*, is responsive to light was reported by Gustafson (1935). Our own interest was aroused by the behaviour of some hags (kindly sent to us by Dr Gustafson) which were being photographed. A tank containing a number of them was brightly illuminated for a few seconds only. Some 20 sec. later the animals, which had previously lain motionless in the semi-darkness, started, rather suddenly, to swim. The long delay between stimulus and response seemed to merit study while the definiteness of the response promised an important help to observation. The present paper reports the behaviour of hags under different conditions of illumination and a preliminary investigation of the sensory physiology of their responses.

METHODS

In two successive years over thirty animals were received from Sweden and from each batch twenty, as uniform in size as possible and with the largest and smallest individuals excluded, were selected for our work. Each of these animals was kept in a separate rectangular tank 24 in. long, 12 in. wide and 12 in. deep under about 3 in. of sea water. No aeration was provided, and the glass floors of the tank were left bare since *Myxine* given mud or gravel will always disappear into it. The tanks were kept in a room from which daylight was excluded and whose temperature stayed between 11 and 14° C.

For the convenience of the observers, and to permit records to be made of the behaviour of animals while in 'darkness' the room was lit by two 15W. red-painted bulbs throughout the period of the observations. Occasionally dim red light from a hand torch was used to supplement the general illumination. The background illumination did not reach 10 ca.m. (candle metres) at the floor of any of the tanks. We shall hereafter refer to animals as being in the dark when their only illumination was from these sources.

To test their responses the animals were illuminated, one at a time, by means of electric light bulbs mounted in a hood which could be rested on top of the tanks. While lit in this way an animal could be watched through the side of its tank. This method does not provide a uniform light intensity over the floor of the tank and to this is due one of the uncontrolled variables in our observations. Another is

a consequence of the different positions adopted by animals when at rest during stimulation by light. They usually lay on their sides, but sometimes lay on their bellies, or even, rarely, on their backs, and the aspect presented to the source of light varied accordingly.

When in the dark under these conditions our hags normally lay quite still. That 'spontaneous' movements do occur was, however, very evident and so before any animal was subjected to a light treatment it was watched for 1 min. in the dark. If during that time it made any visible movement it was deserted for at least 15 min. and then watched for a further period of 1 min. In no case was it necessary to defer an observation on an animal more than twice.

After treatment with light animals were usually allowed a minimum of 30 min. in the dark to recover, but for certain experiments this period was reduced to 15 min.

OBSERVATIONS

(1) *The activity of animals in the dark*

We have two measures of the frequency of 'spontaneous', i.e. non-induced, movement in our animals. In the first place we made a record of each occasion upon which an observation had to be deferred because of movement during the 1 min. pre-treatment period. In the second place we watched each of one batch of animals continuously for 1 hr. in the dark and recorded every spell of activity shown.

Thus on 679 occasions we approached one or other of a group of twenty animals for the purpose of testing its responses to light. Of that number forty-two approaches were deferred because the animal moved during its 1 min. period in the dark, or was moving when first approached. Of the forty-two deferred approaches three represent observations twice deferred. There seem to be real differences between the levels of spontaneous activity of the animals when measured in this way, but there is no obvious correlation between this and the differences in the responses to light which also appeared.

When twenty animals were watched continuously for an hour in the dark ten of them remained still for the whole of that period. Of the other ten, four engaged in a single burst of swimming, five in two separate bursts, and one in three separate bursts. In other words, swimming occurred 17 times in 20 'animal-hours' of observation.

Such movements in animals under observation could, of course, be mistaken for responses to light stimulation. Their effect will then be to produce mean reaction times lower than the correct figures or to indicate a response where none is evoked. This source of error would clearly be serious where the reaction time approached the mean interval between spontaneous movement. As, however, we were always concerned with reaction times of less than 5 min. (and usually less than 1 min.) we can safely ignore this source of error, although we believe that the very few of our measurements of reaction times that are strikingly less than the mean of their kind may be accounted for in this way.

(2) *Response of* Myxine *to general illumination*

The illumination of *Myxine* elicits general locomotory activity which begins sometimes with a local response at the head or tail but more often as a general stirring of the whole animal. These movements are always sharp and follow a period, never less than a few seconds, during which the animal remains still. Active locomotion may take some time to develop after these first stirrings. The hag in the dark usually lies on its side. On illumination it gets on to its belly and then, after intermittent stirring assumes a swimming posture by throwing its body into waves. Soon afterwards the full locomotory response emerges. Once this happens the animal may swim for several minutes, making periodic attempts to burrow. We shall not concern ourselves further with these later phases in the

Table 1. *Reaction times of twenty animals to general illumination at* 344 *ca.m.*

Animal	Time to first response (sec.)					Mean
A	30	34	37	24	22	29·4
B	14	17	14	12	20	15·4
C	6	7	14	9	11	9·4
D	36	22	27	19	17	24·2
E	24	47	56	23	24	34·8
F	20	21	32	14	18	21·0
G	15	12	22	16	20	17·0
H	20	18	13	18	26	19·0
I	15	17	18	21	15	17·2
J	32	33	32	27	23	29·4
K	23	24	34	20	29	26·0
L	8	16	17	21	15	15·4
M	29	19	29	13	27	23·4
N	13	16	14	17	21	16·2
O	33	22	23	18	16	22·4
P	8	14	19	9	18	13·6
Q	13	14	14	13	13	13·4
R	12	13	11	14	11	12·2
S	20	12	13	29	11	17·0
T	28	21	33	43	49	34·8
Total	399	399	472	380	406	—
Mean	19·95	19·95	23·6	19·0	20·3	20·56

behaviour of an animal aroused by light. But the sharpness of the first response makes it possible to use this in measuring reaction-time, and most of our work has been concerned with the measurement of this reaction time under various conditions.

Reaction times of a group of twenty selected animals illuminated 5 times by a 40 W. lamp (mean intensity 344 ca.m. at the floor of the tank) are given in Table 1. From the mean reaction time of about 20 sec. we see that *Myxine* is slow to respond even to fairly intense illumination. Some corresponding figures from other animals at intensities of this order are: ammocoetes, 2 sec. (Young, 1935; Steven, 1950); tadpoles of *Rana clamitans*, 2 sec. (Obreshkove, 1921); *Ciona*, 5–10 sec. (Hecht, 1918); *Mya*, 1·3–1·5 sec. (Hecht, 1919*a*). Only *Proteus* (Hawes, 1945) has a comparably slow response.

Table 1 also shows how much the reaction time can vary under our experimental conditions, even when the light source remains the same. This variation may be

due in part, and perhaps in the main, to differences of intensity in different parts of the tank and differences in the lighting of an animal according to the way it lies. However, the means of the separate series of the entire group do not depart far from the general mean for the five series taken together.

It is also clear from Table 1 that there is a big variation in reaction time from one animal to another, some are clearly 'fast' and others 'slow'. Thus animals E and T gave mean reaction times 3–4 times as long as C or R. For this reason, in certain work to be described later, we confined observations to a few, and in some cases to single, animals whose performance had been established as relatively consistent.

Intensity/time data

It was to be expected that the response of *Myxine* to light would vary with the intensity of illumination, and tests showed that though the character of the response is the same over the whole range of intensities that give responses at all, the reaction time varies inversely with intensity.

Our data on the effects of varying intensity on the reaction time fall into three groups: (1) results on twenty selected animals whose reaction times at a single intensity were given in Table 1; on these we only obtained three readings for each animal at each of four intensities; (2) results on five animals with eight readings at each of five intensities; (3) results on one animal with twenty-five readings at each of seven intensities. The results of each of these are given graphically in Fig. 1 *A–C*, and the numerical data for Fig. 1 *C* are given in Table 2. The picture is the expected one. At high intensities the reaction time approaches a minimum and, in this region, big increases in intensity have little effect upon it. At lower intensities the reaction time is a matter of minutes and it becomes difficult to be certain that one is not dealing with a spontaneous movement.

Strict adherence to the Bunsen–Roscoe Law is not to be expected, since we know from Hecht's work (1918, 1919*a*) on *Ciona* and *Mya* that in them reaction time in a response to light is made up of two components, of which one alone varies with intensity in the Bunsen–Roscoe manner. Thus if we extract from Table 2 the data on the three lowest intensities we find that the products of reaction time and intensity are 162, 146 and 131 approximately, and not nearly as constant as the Bunsen–Roscoe Law demands.

Sensitization time and latent period

As in the response to light in other animals, it is not necessary to illuminate *Myxine* for the full period of the reaction time to get a normal response. It is clear, therefore, that the reaction time is composed of the two components of Hecht mentioned above, sensitization-time—the period of exposure to light necessary for response in minimum time; and latent period—during which the animal need not be illuminated. We tested the reaction times of our groups of twenty animals with illumination of limited duration, and the results are set out in Table 3. One can reduce the period of illumination to 5 sec. at this intensity (344 ca.m.) without any reduction in the reaction time. If further reduced to 1 sec. most of the animals still

respond but only after a much longer time. Even with a flash of approximately 0·1 sec. duration, ten out of the twenty animals responded, usually from 2 to 5 min. later.

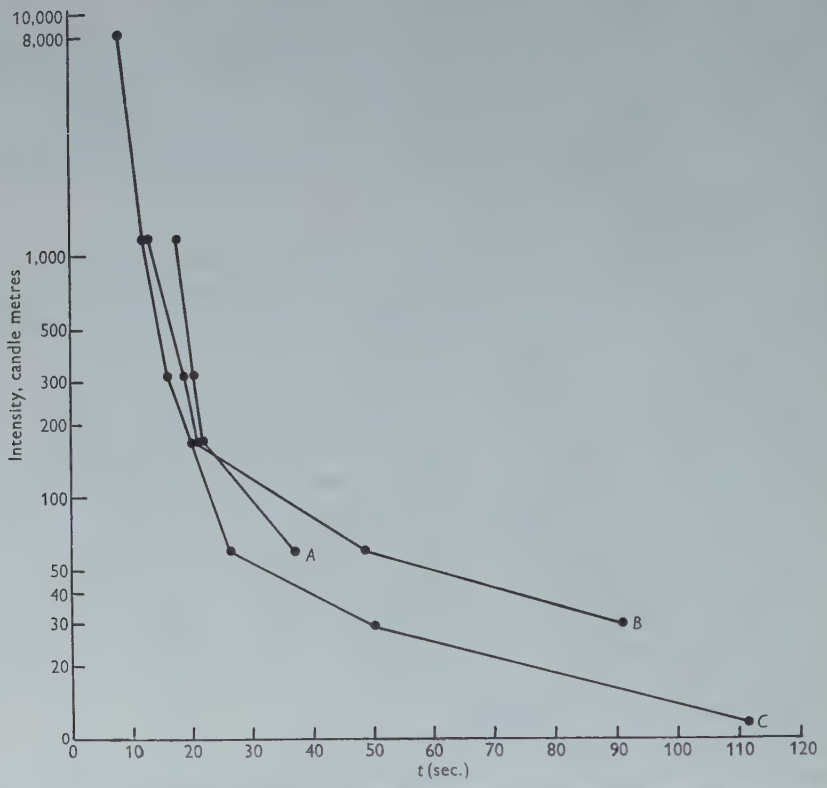

Fig. 1. The relation between reaction time and intensity. *A*, means of twenty animals and three readings at each intensity; *B*, means of five animals and eight readings at each intensity; *C*, means of twenty-five readings at each intensity on a single animal.

Table 2. *Reaction times of animal F to general illumination at different intensities. Means of twenty-five observations at each intensity with standard deviations*

Intensity (ca.m.)	Mean reaction time (sec.)
8650	8·8 ± 1·60
1258	12·3 ± 1·65
344	16·3 ± 2·18
184	20·5 ± 2·06
65·6	26·5 ± 4·06
31·2	50·4 ± 10·4
12·6	111·7 ± 30·0

It was important to determine how much of the reaction time was taken up with sensitization and how much with latent period over a wide range of intensities, and especially in the long reaction times with low intensities. We did this simply by

finding the shortest periods of illumination that would still give reaction times of minimal value at several intensities.

Our most complete data on sensitization are based on three animals only, and for one of these sensitization was determined at a number of different intensities. The first two of these animals yielded the results given in Table 4. Inspection of these results indicated a sensitization time of 5 sec. in both cases, with latent periods of 21 and 22 sec. Here the latent period is by far the longer of the two components, and most of the long reaction time is due, not to the photochemical process, but to the time taken by the products of the photochemical reaction to initiate the response. It is a little unfortunate that these two animals had almost identical mean reaction times so that they cannot tell us whether differences between animals are to be

Table 3. *Reaction times of twenty animals to general illumination at* 344 *ca.m. with varying exposure periods*

Duration of illumination (sec.) ...	15	10	5	1	Flash 0·1 sec. approx.
Mean reaction-time (sec.)	31·25	31·55	31·84	72·06	177·22
No. of animals responding	20	20	19	16	9

Table 4. *Reaction times (sec.) of animals D and N to general illumination at* 344 *ca.m. with varying exposure times (means of* 20 *tests)*

Duration of illumination	Until roused	10	9	8	7	6	5	4	3	2	1
RT Animal D	26·0	24·2	—	—	—	—	22·9*	36·5	40·1	45·6	56·5
RT Animal N	26·8	24·4	26·1	27·6	25·4	28·5	29·6*	55·8	—	—	—

* Probable sensitization period.

attributed to differences in sensitization period or latent period. Our third animal, whose performance at different intensities is examined below, can contribute something on this point. At the intensity (344 ca.m.) used in determining sensitization in the other animals it had a reaction time of 16 sec. and a sensitization period of 6 sec. This result suggests that sensitization time is roughly constant from animal to animal and that it is the latent period that is responsible for the big and consistent differences between animals that were noted in an earlier section.

Table 5 consists of sensitization data from animal F at five different intensities. Five readings of reaction time were taken for each exposure at each intensity. The simplest way of expressing sensitization time from the figures is to take it as the shortest of the exposure times giving a reaction time not appreciably longer than the reaction time when the light remains on until the animal is roused. At 344 ca.m. this would be 6 sec., though it is not until one reduces the exposure time to 3 sec. that all the reaction times in a series exceed the minimal figure. Perhaps the halfway position of 4·5 sec. between the exposure time when delayed responses first

appear (6 sec.) and that when all responses are delayed (3 sec.) would give a closer approximation to the true sensitization period. However, the sensitization periods given in the table for the five different levels of intensity are those obtained by the method of inspecting the means and picking out the last one that still gives near-minimal reaction times.

The figures show that while the highest intensities of all failed to speed up sensitization period as much as one might have expected, the effect is very clear at moderate and low intensities. The latent period varies much less, but it is not constant and on this we shall comment in the discussion. Thus *Myxine*'s comparatively slow reaction at higher intensities is mainly taken up by the latent period. But the exceptionally slow reactions at the lower intensities are mainly due to the time required for sensitization.

Table 5. *Reaction times, sensitization periods and latent periods of animal F at five intensities. Reaction times are means of five readings*

Intensity (cm).													Sensitization period	Latent period
1258	Exposure time	UR*	5	4	3	2	.	.	.	.	.	.	5	9·8
	Reaction time (sec.)	14·8	16·2	20·8	26·0	†	.	.	.	.	.	.		
344	Exposure time	UR*	10	9	8	7	6	5	4	3	2	.	6	9·8
	Reaction time (sec.)	15·8	15·6	15·8	15·6	15·8	16·2	18·6	20·2	25·6	†	.		
184	Exposure time	UR*	15	14	13	12	11	10	.	.	.	.	14	11·4
	Reaction time (sec.)	25·4	24·6	24·2	31·6	32·0	37·2	†	.	.	.	.		
65·6	Exposure time	UR*	28	27	26	25	24	23	22	21	20	19	26	11·0
	Reaction time (sec.)	37·0	35·8	41·4	41·6	57·8	46·8	51·8	59·6	78·0	58·2	†		
31·2	Exposure time	UR*	44	42	40	38	36	34	.	.	.	.	42	16·2
	Reaction time	58·2	64·0	52·4	76·8	81·2	†	†	.	.	.	.		

* Until roused.
† Less than five responses obtained.

At the same time we have an example of the prolongation of the reaction time by illuminating for periods far below the sensitization period. Indeed it is difficult to get down to an exposure time at any intensity when the reaction fails altogether. When Hecht encountered this effect in *Mya* (1919*b*) and in *Ciona* (1926) he overlooked, in our opinion, certain of its implications. He simply regarded it as the lengthening of the latent period. For reasons that we shall go into later we find it unsatisfactory to apply the term latent period to the very different conditions that exist when exposure time falls far short of the sensitization period. What is noteworthy in this effect is the essential unity of the sensory process in spite of its two stages. Thus one can get the same result in terms of reaction time from a very brief exposure at a high intensity as one gets from prolonged exposure at a lower one.

Other factors affecting reaction time

A number of factors besides intensity influence reaction time. As might be expected, temperature has an effect. Thus an animal that reacted to light at 344 ca.m. after 36 sec. at 12·2° C., reacted after 62 sec. at 6·7° C. Another which reacted at 14 sec. at 11·7° C. did so after 32 sec. at 4·0° C. Such a sensitivity to temperature again indicates the large proportion of the reaction time that is taken up by non-photochemical reactions.

Exposure to light also has the effect of slowing down the responses for some time afterwards, and if the illumination is continued for a long time, e.g. half an hour, the animal can be made temporarily altogether insensitive. The return of sensitivity after such treatment is slow, as we can see from the following experiment on ten hags. These were exposed to continuous illumination at 344 ca.m. for 30 min. Then after 2 min. in the dark only one animal responded to a period of illumination lasting 2 min. After 5 more minutes in the dark, 2 animals responded, after 10 further minutes nine animals responded; after 15 more minutes again only nine

Table 6. *Reaction times of animals F and N (means of five readings) before and after illumination at* 344 *ca.m. for* 1 *min.*

	Animal F (sec.)	Animal N (sec.)
Before illumination	13·7	28·7
1–3 min. after illumination	25·0	47·6*
3–6 min. after illumination	17·2	30·7
6–9 min. after illumination	15·6	26·9
9–12 min. after illumination	14·2	29·1

* Mean of three readings only.

responded and it was only after another 30 min. in the dark that all ten had recovered their sensitivity to light. The mean reaction times of the animals responding after 10, 15 and 30 min. in the dark were 117, 67 and 54 sec. respectively, compared with 18·1 sec. at the beginning. Clearly a long time in the dark is required for return to normal sensitivity after such treatment.

The effect of shorter periods of illumination on reaction time was also investigated. The animals were illuminated for 1 min. and then the reaction times to further exposures after certain times had elapsed were measured. As readings could not be taken until the animals had come to rest, it was not always possible to do tests within 3 min. of the first exposure. The results from two of these animals are given in Table 6. They show that it can be assumed that reaction time is back to normal, in other words, that dark adaptation is complete, within a few minutes after exposures of the kind used in our experiments, and that an interval of 15 min. between successive readings was long enough to permit comparable results to be obtained.

Surprisingly, the reaction to light is delayed if the animal is already active when the illumination begins. Thus if a hag is roused by sharp tactile stimulation and

then illuminated while it is still swimming or in a swimming posture, the response to light follows much later than in the resting animal. Ten such tests were carried out with one of our animals and gave the following results: mean reaction time of ten controls at 344 ca.m. illuminated at rest—20·3 sec., mean reaction time of same ten animals when illuminated while response to tactile stimulation still in progress—40·2 sec. It may seem strange that one can still determine the reaction time to light under these conditions. In fact the activity induced by a tactile stimulus was usually sufficiently short-lived for the hagfish to relax completely after the light was turned on. Then after lying still for a time, the typical response to light developed. Evidently the reaction time to a light stimulus can be lengthened by the central nervous system. This is a warning against believing this kind of behaviour to be automatic and invariable.

Response to local illumination

By using Perspex rods and a 36W. light source it was possible to explore the surface of the animal to find out where the light sense is located. As a preliminary, twenty animals were examined with a rod 3·8 × 0·8 cm. in cross-section. Fig. 2*a* shows the areas illuminated by this rod, the head, the branchial apertures, the cloaca and the tail. All responded to local illumination on the head, the tail and the cloaca, but at the branchial apertures only ten responses were obtained. The intensity on these illuminated patches was 790 ca.m., and the reaction times somewhat longer than with general illumination of this intensity. It seemed that *Myxine* had regions of high sensitivity to light anteriorly and posteriorly, and areas of lesser sensitivity in the mid-body region.

With rods which illuminate smaller patches it was possible to delimit the light-sensitive areas more precisely. A rod 2 × 0·8 cm. in cross-section gave the following picture on the same group of twenty animals:

Anterior head	20 responses
Posterior head	10 ,,
Branchial apertures	2 ,,
Midway branchial apertures and tail	2 ,,
Cloaca	19 ,,
Tail	7 ,,

Using a third rod of dimensions 1·05 × 0·8 cm. in cross-section two hags were explored along the whole of their lengths. The results are shown in Fig. 2 on the outline drawings of these animals. Moving the rod along the body gave twenty-six bands 0·8 cm. wide in animal D and twenty-four in animal F. Each band was illuminated 5 times. Except where there are definable landmarks, e.g. branchial apertures, tail tip, cloaca and mouth, the location of the bands varied a little from one series of readings to another, but it so happens that it was these most easily marked regions that proved to be most important. The number of responses to the five tests is indicated in each band on the drawing and the mean reaction time below.

These results show that *Myxine*'s light sense is located mainly near the anterior end and in the cloacal-caudal region. Probably *Myxine* has light-sensitive end-organs over most of its body, which are dense at certain places in the head and in the cloacal and tail region, but sparse elsewhere. Presumably, if local illumination is to rouse the animal a minimum number of these end-organs must be excited. Thus light from the smaller rods fails to rouse if directed anywhere between points just posterior to the mouth and anterior to the cloaca. Even in the more sensitive areas, the reaction to light fails if only very tiny areas are illuminated. Thus when

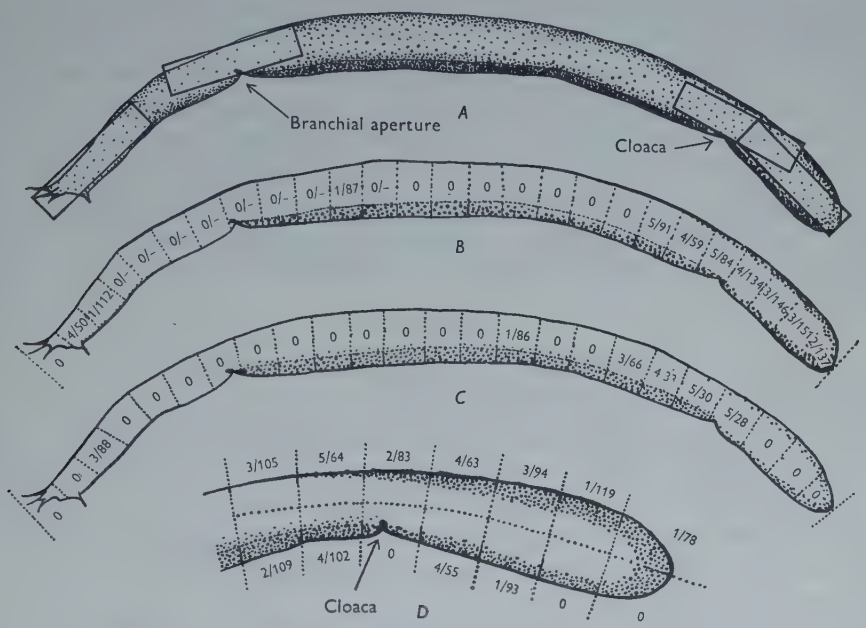

Fig. 2. Local illumination of *Myxine*. *A*, areas illuminated with rod 3·8 × 0·8 cm. cross-section; *B*, areas illuminated on animal D with rod 1·05 × 0·8 cm. cross-section; *C*, areas illuminated on animal *F* with rod 1·05 × 0·8 cm. cross-section; *D*, areas illuminated on posterior region of animal D. In *B–D* and *D* the figures refer to the number of responses obtained in five trials and to the mean reaction time in seconds where responses occurred.

the skin was explored with a rod 0·4 × 0·4 cm. in cross-section, no response occurred even when this was directed on to the sensitive places in the head and near the cloaca.

The region of the cloaca and the tail is the most extensive area of high sensitivity to light. In the ammocoete the main light-sensitive organs occur in the tail (Parker, 1905; Young, 1935; Steven, 1950), but in *Myxine* the immediate vicinity of the cloaca is the most sensitive place of all. Tests using a smaller rod showed that the lips of the cloaca itself are not light sensitive. This point is illustrated by Fig. 2*D* in which the results of separate dorsal and ventral illumination of the last 7 bands into which this animal (D) may be divided are shown on an outline drawing.

The light-sensitive area in the head is more restricted in extent, apparently not more than 0·8 cm. wide in either of the two animals D and F. While these, and most

other animals, gave no sign of sensitivity to light at the extreme anterior end, we did encounter one remarkable animal which always responded to local illumination here within 4–8 sec.

The highly sensitive region in the head is well in front of the vestigial eyes. While it might almost be assumed on general anatomical grounds, e.g. the poor optic nerves, that the eyes are not involved in the response, it seemed wise to test the matter. The eyes of two hagfish were destroyed under urethane anaesthesia. Both continued to respond to local illumination on the head. This, and the location of the light-sensitive region in the head, shows that the light sense here is non-optic.

It is generally true that our hagfish reacted more quickly to general than to local illumination. But perhaps more important than the quantitative differences in the reaction time are certain qualitative differences in the kinds of reactions resulting from the two kinds of illumination. While it is true that many animals gave the typical response after local illumination at the posterior end, in many other cases they made only local responses, a flexing of the tail or some other movement that did not involve the rest of the body. These local movements were a common feature of the response; and they always took the same form in those animals which showed them.

The role of the nervous system in the response of Myxine *to light*

It has been implied in the earlier sections that the response to light takes its origin from specifically light-sensitive end-organs situated in the skin. The possibility that changes in light intensity are acting directly on the central nervous system cannot however be ruled out, especially in view of Young's (1935) evidence for certain responses of ammocoetes to light—and relatively slow ones at that—being due to the light acting directly on the spinal cord. Indeed the fact that *Myxine* is most sensitive to light at the head and near the tail, is consistent with direct excitation of this kind since these are the places where the central nervous system is nearest the body surface.

Two kinds of experiment were carried out to test this matter. In one, a length of brain or spinal cord was exposed and its sensitivity to direct illumination was tested. In the other, the skin was removed over several centimetres in front of the cloaca and the sensitivity to light within this skin-free area compared with that of the light-sensitive region just behind it. In these tests, the animals were, as usual, immersed in sea water.

In the first experiments the brain and the anterior end of the spinal cord were exposed and then illuminated locally with our Perspex rods in the usual way. If we are dealing with direct effects of light on the spinal cord and brain, it would be fair to assume that the removal of the skin and other tissues covering these parts of the central nervous system would increase the effective intensity of the incident light and so reduce the reaction time. In the event the two animals tested in this way had reaction times to illumination on the operated region that were more than doubled by the operation.

Table 7 shows the results of the experiments in which the skin was removed from

an area just in front of the cloaca known to be highly sensitive to light before the operation. The figures show quite clearly that the light sense goes with the skin. We are satisfied that the response to light in *Myxine* cannot be attributed to direct stimulation of any part of the central nervous system.

The chief neurological problem raised by this demonstration of photo-sensitivity in the skin at the posterior end lies in establishing the sensory pathways from the photo-receptors to the central nervous system. Added interest is attached to this problem in *Myxine* by the fact that the light receptors in the tail of *Lampetra* have

Table 7. *Responses of* Myxine *to local illumination before and after removal of skin anterior to cloaca*

Before operation						After operation					
Region for skin removal			Region to be left intact			Skin-free region			Skin intact		
No. of trials	No. of responses	Mean *RT* (sec.)	No. of trials	No. of responses	Mean *RT* (sec.)	No. of trials	No. of responses	Mean *RT* (sec.)	No. of trials	No. of responses	Mean *RT* (sec.)
24	24	25·3	23	23	41·7	29	4	155·2	31	26	56·1

Table 8. *Responses of* Myxine *to local illumination after transection of the spinal cord*

Head illumination			Cloacal-caudal illumination		
No. of trials	No. responses from head	No. responses from tail	No. of trials	No. responses from head	No. responses from tail
36	36	0	50	2	46

Table 9. *Responses of* Myxine *to general illumination after transection of the spinal cord*

No. of trials	No. responses from head	No. responses from tail	Mean *RT* head (sec.)	Mean *RT* tail (sec.)
33	33	32	42·4	35·1

been shown to be innervated through the lateral-line nerves to the tail (Young, 1935). This is shown in the ammocoete by the fact that the head alone responds when the tail is illuminated after transection of the spinal cord. This response disappears when the lateral line nerves are severed.

The spinal cord was transected under anaesthesia in two groups of five hagfish in successive years. The results appear in Table 8. It can be seen at a glance that the situation in *Myxine* is quite unlike that in the ammocoete. In spinal *Myxine*, if the head is illuminated, only that part of the animal anterior to the cut responds; posteriorly the animal remains quite passive. If the cloacal-caudal region is illuminated, only that part of the animal posterior to the cut responds. The situation is revealed equally clearly when spinal animals are exposed to general illumination.

Then, as Table 9 shows, independent responses occur in the two halves of the animal. In the spinal ammocoete, general illumination only causes responses of the head.

Thus the spinal cord in *Myxine* provides the pathway along which impulses from the photo-receptors near the cloaca travel anteriorly to the brain. But even when the posterior part of the body is isolated by spinal section, local responses are still possible through local reflex arcs which take slightly different forms in different individuals. There is none of this in the ammocoete. This evidence that the spinal nerves conduct the excitation from the photo-receptors to the central nervous system reveals an arrangement hitherto not known with certainty in a vertebrate. For while it is true that other cases of sensitivity to light in the skin of vertebrates (blinded tadpoles, *Proteus*, blind fish, etc.) may well be due to innervation through

Table 10. *Responses of* Myxine *to local illumination before and after severance of spinal nerves anterior to cloaca*

Before operation						After operation					
Region of operation			Region to be left intact			Operated region			Intact region		
No. of trials	No. of re-sponses	Mean *RT* (sec.)	No. of trials	No. of re-sponses	Mean *RT* (sec.)	No. of trials	No. of re-sponses	Mean *RT* (sec.)	No. of trials	No. of re-sponses	Mean *RT* (sec.)
15	15	24·8	15	15	37·2	21	2	78·0	21	18	70·7

the spinal nerves, this has not been established. In an additional experiment we exposed a length of spinal cord and stripped it of all its connexions with surrounding tissues severing ventral as well as dorsal roots. The operated region was usually taken just in front of the cloaca, or even anterior to that if such a region proved to be sensitive to light in preliminary tests. After the operation the sensitivity of the operated region was compared with the intact cloacal-caudal region just behind. The results obtained from four animals are given in Table 10. The skin of a region operated upon in this way loses its sensitivity, but there is a good deal of spontaneous activity after such drastic operations and this, in our opinion, explains the inconsistencies in the results. This is additional evidence that the spinal nerves serve the photo-receptors of the cloacal-caudal region.

Nothing has been done, because of the technical difficulties involved, to trace out the innervation of the much more restricted light-sensitive region in the head. However, in some of our animals it must be said that the head is the more sensitive of the two regions. Several of them had to be rejected for some of the experiments which required high sensitivity to light in the posterior region because only the head responded to the local illumination used in these tests.

It would seem that photoreception in *Myxine* is a general skin sense subject to individual variation in sensitivity and distribution, its connexions with the central nervous system are provided by the sensory roots of the spinal nerves in the posterior region, and probably by analogous sensory fibres in one of the cranial nerves in the head.

DISCUSSION

Myxine provides an example of a non-visual reaction to light which apparently conforms to the general principles derived by Hecht from work on more rapidly responding invertebrates. These are that the reaction time is composed of a photo-

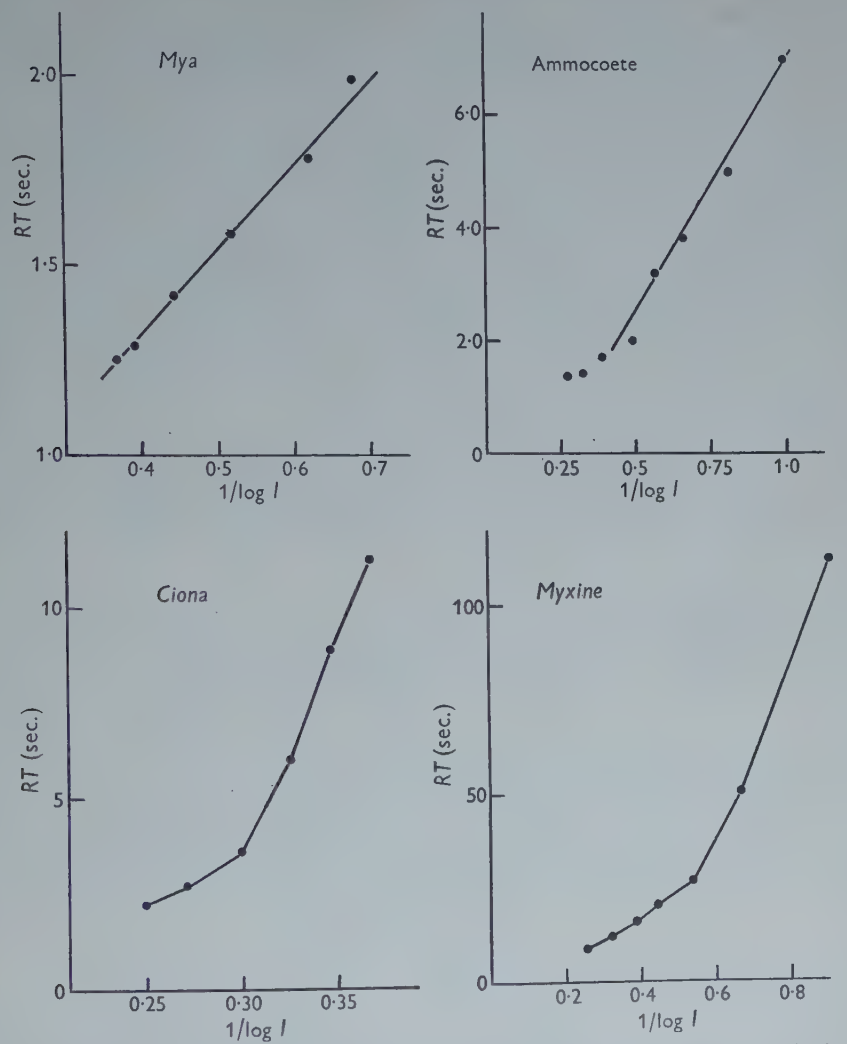

Fig. 3. The relation between the reaction time and the reciprocal of the logarithm of the intensity in *Mya*, ammocoete, *Ciona* and *Myxine*. The curves for *Mya* and ammocoete are redrawn from Hecht (1921), fig. 2, and Steven (1950), fig. 4, respectively. The curve for *Ciona* is constructed from Hecht (1918), table 2. The curve for *Myxine* is constructed from the data of Table 2 of this paper.

chemical phase, whose intensity/time relations are roughly in accordance with the Bunsen–Roscoe Law, and a non-photochemical phase appearing as a latent period, taken up with certain secondary processes. In considering our results with *Myxine*,

however, we have found it necessary to modify the conception of the latent period originally proposed by Hecht.

Hecht's latent period is not a quantity that can be measured directly. It is a difference, a quantity obtained by subtracting a period of exposure to light from a total reaction time. We have seen that in *Myxine*, as in *Ciona* and *Mya* on which Hecht worked (1918, 1919*a–c*), there is a certain critical exposure time, the sensitization period, beyond which further exposure does not shorten the reaction time at any given intensity. At this exposure the reaction time is minimal, and according to Hecht (1918) the latent period obtained by subtracting the sensitization period from the reaction time is constant in *Ciona* over a considerable range of intensities. We were a little surprised, therefore, when our own values for the latent period in *Myxine*, with full sensitization, were not constant over our range of intensities. They went from 15·2 sec. at the lowest intensity (31 ca.m.) to 9·8 sec. at the highest (1258 ca.m.). With even more intense illumination (8650 ca.m.) the reaction time, of which the latent period is a part only, fell to a mean value of 8·8 sec. Our determinations of the latent period were only rough approximations, yet the progressive and consistent shortening of this difference between critical exposure time and reaction time needs explanation.

Further consideration suggested, however, that a constant latent period with varying intensity is only to be expected when sensitization is almost instantaneous as in *Mya*. Indeed this follows from Hecht's own conception of the nature of the photosensory process, as a coupled photo-chemical reaction in which, to quote his (1919*b*) summary:

> The events which happen in the sense organ of *Mya* when it is stimulated by light may, according to our findings, be expressed as follows. The photosensitive substance (S), originally formed from its two precursors (P and A—Precursor and Accessory), is changed back into them under the influence of light, both reactions being given by the expression $S \underset{\text{dark}}{\overset{\text{light}}{\longleftrightarrow}} P-A$. This happens during the exposure to light or during the sensitization period when the exposure is prolonged. One or both of the freshly formed precursor substances then immediately serves to catalyse the transformation of an innocuous material (L) into a stimulating substance (T). This occurs during the latent period. When a sufficient amount of the stimulating substance (T) has been accumulated, it acts on the nervous connections to the sense organs and initiates the retraction of the siphon. The entire sensory process may therefore be summed up in the following two reactions:
>
> $$S \rightarrow P+A; \quad L//P+A// \rightarrow T,$$
>
> in which the symbol $//P+A//$ signifies catalysis by one or both of the precursor substances.

The weakness of this conception lies in its assumption that the latent period and the reaction $L \rightarrow T$ are co-extensive in time. In practice this reaction will surely not wait until the completion of the photochemical reactions $S \rightarrow P+A$ before itself beginning, and it seems to us better to regard these two reactions not as consecutive but as contemporary or, at least, overlapping. The latent period is then not the entire duration of the reaction producing T but simply the time required, over and above the sensitization period, to produce T.

If this is so then changes in intensity will affect not only the sensitization phase but through it the latent period also. Hence one may expect the latent period to

change with intensity in a way which while unimportant where sensitization is very brief (e.g. in *Mya*) is marked where this is relatively long (e.g. in *Ciona* and *Myxine*).

A further complication, which we do not profess to explain, must be the cause of a feature of the intensity/time relationship in *Ciona*, the ammocoete and *Myxine*, which has not before received attention. For if the sensitization process obeys the form:

$$\text{Photochemical effect} = k \times \text{duration of stimulus} \times \log \text{intensity},$$

where k is a constant, then the curve of reaction time against the reciprocal of the log intensity should be a straight line cutting the time axis at a value corresponding to the latent period where this is a constant. In the case of *Mya* the data fit this expectation well, but inspection of Steven's (1950) curve for the ammocoete shows that while the relation is linear for low intensities it is impossible to derive a real value for the latent period from this part of it. Hecht's figures for *Mya* and *Ciona*, Steven's for the ammocoete and our own for *Myxine* are plotted in Fig. 3. In *Ciona* and *Myxine* the upper (low intensity) part of the curve is, or may well be regarded as, straight; but if produced cuts the time axis at a negative value. The lower (high intensity) part departs quite markedly from the linear. Steven's data are quite consistent with a similar situation in the ammocoete, although he did not work with intensities sufficiently high to bring the animals well into the non-linear part of their reactions. If, then, Hecht's picture of the reaction time only provides an adequate description of events where the sensitization period is short compared with the total reaction time, our own modification is still unable to explain the actual situation in *Ciona*, the ammocoete and *Myxine* where sensitization is relatively long. We do not think that the data at our disposal are sufficiently accurate and detailed to make it worth while fitting a deduced curve to them and we must reluctantly leave this matter unexplained.

We may here comment briefly on the biological significance of *Myxine*'s light sense. Parker (1909) believed that dermal photo-reception was found only in those fish that were predominantly fresh water in habitat, and he was driven by this to revise his earlier views on the evolutionary origin of the vertebrate eye. Here it is only necessary to point out that *Myxine*, by robbing Parker's premise of its general validity, must weaken his argument from it.

From the comparative point of view it is of some interest to discover that though each of the groups of living cyclostomes is light sensitive at the tail end of the body, the innervation of the sense organs is different in each case. This makes it possible that the light sense was evolved independently in lampreys and hags. The alternative is that one mode of innervation replaced the other in one of the two groups, but we think it more likely that the two senses are of independent origin and testify to the ability of the vertebrate skin to evolve photoreceptors and the peripheral nervous system to innervate them in situations in which they meet the needs of the animal. Possibly the light sense in *Myxine* developed in animals that lacked a lateralis system in the tail and trunk and hence could only be innervated by spinal nerves, while in lampreys a fully functional lateralis system permitted an alternative

nervous pathway. It would certainly be of great interest to know whether lateral line nerves do serve dermal photoreceptors in any other aquatic vertebrates.

There are obvious differences, however, in the relative importance of the light sense to the lampreys and hags. The light sense of the lamprey tail has an important and well understood role to play in the life of the ammocoete at least, but it is possible to doubt the value of *Myxine*'s sense both because it is so feeble and because many *Myxine* are caught in water so deep that it must there be inoperative. We are not, however, inclined to dismiss the light sense as a functionless evolutionary survival in *Myxine*, as Hawes felt bound to do in the rather different case of *Proteus*. The matter is discussed fully by Steven (1955) on the basis of his findings on the spectral sensitivity of the light sense, but for our part we feel that the very localization of the sense points to a continuing role of light perception in the life of those hags inhabiting, or visiting, relatively shallow waters. Its effect would seem to be, if we argue from behaviour in the laboratory, to keep the hag buried during daylight hours and in particular to ensure that the tail as well as the head is withdrawn beneath the surface of the sea-bed. It may also be expected to deter the movement of hags from deeper to shallower waters, but discussion of such points only emphasizes our ignorance of *Myxine*'s normal mode of life.

SUMMARY

1. *Myxine glutinosa* responds to illumination by active locomotory movements.

2. The response to light occurs some time after the onset of illumination. This time can be resolved, after the method of Hecht, into a sensitization period and a latent period.

3. Analysis of the relation of sensitization period and latent period to intensity of illumination and other factors shows that photoreception in *Myxine* is essentially similar to that of a number of other animals, including the ammocoete, but suggests that the secondary reactions initiated by the production of photolytes during sensitization occur during both sensitization and latent periods and not during the latent period alone.

4. The photoreceptors of *Myxine* are located in the skin and are present only, or mostly, at the anterior end of the head and in the region of the cloaca. Nervous impulses travel from the posterior photoreceptors through spinal nerves to the spinal cord.

It is with the greatest pleasure that we acknowledge our indebtedness to Dr G. Gustafson, whose kindness in providing us with animals made our work possible. We have also to thank, for assistance and advice, Dr D. M. Steven, Dr G. P. Wells and Miss R. Birbeck. A grant to one of us (D.R.N.) from the Central Research Fund of the University of London met some of the cost of this work.

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EXPERIMENTS ON THE LIGHT SENSE OF THE HAG, *MYXINE GLUTINOSA* L.

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INTRODUCTION

The general properties of the light reaction of hags have been described by Newth & Ross (1955) in another paper in this *Journal*. They show that although the time interval between the stimulus and the response is exceptionally long, the reaction is similar to those of other eyeless animals such as *Mya*, *Ciona*, *Proteus* or the ammocoete larva of *Lampetra*, and appears to conform in most respects with other photosensory systems. The experiments described in this paper were performed on some of their animals and were intended primarily to obtain information on the hag's spectral sensitivity, from which we may hope to deduce something of the nature of the underlying photochemical system. Additional information is presented on the relation between the duration and intensity of stimulation and the reaction time.

MATERIAL AND METHODS

The experiments were carried out between March and June 1952. They fall into two groups; the first with five hags was done at University College, London, the second with three some weeks later at Edinburgh. All except one of the animals had been used previously by Newth & Ross, and in the first group of tests care was taken to select those which had consistent records of behaviour in their experiments.

Table 1. *Identification of animals*

Experiment series	Animal no.	No. in Newth & Ross's experiments
Edinburgh	1	Not known
	2	Not known
	3	Not known
University College, London	4	B
	5	C
	6	H
	7	L
	8	Not used

The identification of the hags used by them and by me is given in Table 1. It is perhaps worth noting that nos. 4–7, which correspond with Newth & Ross's B, C, H and L, were all fast reacting hags. That is to say their mean reaction time was less than the mean of the whole group of twenty animals in their experiments. No. 5 (= C) consistently gave the shortest reaction time of all.

All experiments were done at room temperature. At University College this varied from 6·5 to 8·5° C.; at Edinburgh from 13·0 to 17·5° C. The latter are probably rather higher than *Myxine* normally encounters in the wild state, but no obvious signs of distress were noticed, and there seems no reason to believe that the animals' light reactions were impaired for this reason. They did not eat throughout the period of these experiments, the last of which were completed about 4 months after the arrival of the animals in this country from Sweden. Again, there is no evidence that their sensitivity to light was affected by this long fast or even that their general condition deteriorated in any way, except that towards the end they seemed to produce rather less mucus when handled.

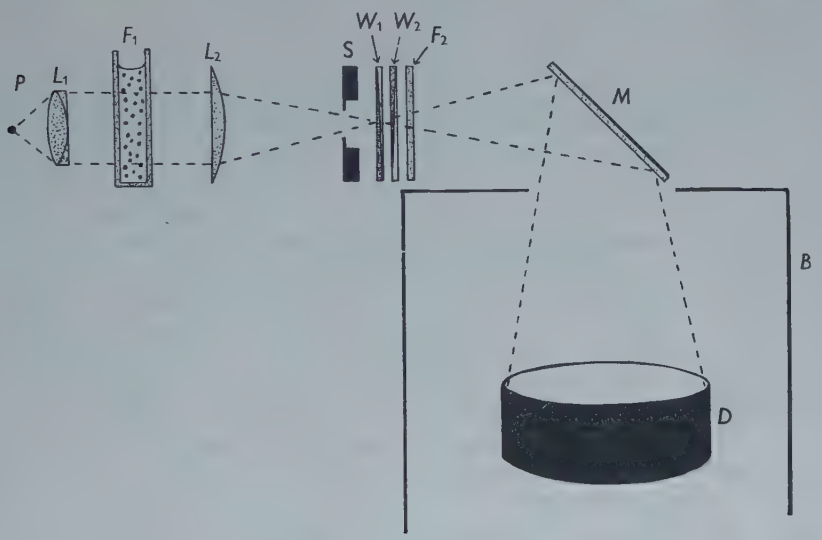

Fig. 1. To show the optical arrangement used for light stimulation of *Myxine* (not to scale). Key to lettering: *B*, dark box surrounding observation dish; *D*, glass observation dish; F_1, heat filter; F_2, colour filter; L_1 and L_2, convex lenses; *M*, mirror; *P*, light source; *S*, camera shutter and diaphragm; W_1 and W_2, neutral wedges.

The apparatus used for light stimulation was in general similar to that described by Steven (1950) for testing ammocoetes, and will not be described here in detail. The optical arrangement is represented diagrammatically in Fig. 1. Light from a source of high intensity was projected so as to illuminate from above a circular glass observation dish 30 cm in diameter. The stimulating light was switched on and off by a camera shutter and its intensity varied by means of a neutral wedge of graded density from 0 to 6, which could be moved horizontally across the path of the beam. These and colour filters when required were mounted close to the principal focus of the lens L_2. A 12 V. 24 W. tungsten filament lamp was used as light source in the experiments at University College, while at Edinburgh a higher range of illumination was obtained with a 110 V. 500 W. projector lamp at a colour temperature of about 3100° K.

The hag to be tested was placed in the observation dish in about 2 in. of clean sea water. White paper was placed under and around the dish, and the apparatus was adjusted so that the cone of light just filled its mouth. This arrangement gave very uniform illumination on the floor of the dish. At University College the experiments were carried out in a satisfactory dark room, and it was necessary only to erect a screen between the lamp and the dish to eliminate stray light. At Edinburgh the room was dimly illuminated and the observation dish was therefore placed in a tent made of cardboard mounted on a wooden frame and painted black on the inside, to which the stimulating light was admitted through an aperture in the roof. A dark cover was placed over the dish between tests so that recovery took place in total darkness.

The procedure for testing was as follows:

The light was switched on and the setting of the wedge and filters adjusted with the camera shutter still closed and the observation dish covered. The cover was then removed and the animal examined for a few seconds in dim red light from an electric hand torch fitted with a filter, Ilford no. 609, which only transmits radiation of wave-length longer than 640 mμ. If the hag was seen to be lying quite still the camera shutter was opened and a stop-watch started simultaneously. The hag was watched continuously, first by the stimulating light and afterwards by the illumination of the red torch, and the times noted of its first movement and other movements up to the commencement of swimming. Animals were usually watched up to 5 min. from the commencement of a stimulus and sometimes longer. If no movement was seen the cover was replaced on the dish and a further recovery period in total darkness allowed before the next stimulus was given.

Twenty to thirty minutes were allowed for recovery between tests at University College, but at Edinburgh periods of an hour or more were given and appeared to be followed by more consistent results. This is at variance with the opinion expressed by Newth & Ross that 20 min. is sufficient for complete dark adaptation of the hag following stimulation. No controlled experiments were done to settle this point, but the impression was formed that longer periods are required to attain maximum sensitivity.

Animals under test were kept in the same dish until a series was completed, sometimes for several days. Lumps of coagulated mucus, the only symptoms observed which might be interpreted as signs of distress, were removed and the water changed if it appeared to be at all dirty. The temperature of the water was measured after each test and at the beginning and end of each series.

The time relation between the first response and swimming

As Newth & Ross have pointed out, *Myxine* responds to light by swimming, but this is nearly always preceded by some other movement, which may be either local or general, and which may be designated the 'first response'. This may be anything from a twitch of the head or tail, or of one or two of the sensory tentacles around the mouth, to a general stirring of the whole body. Sometimes the general movements have the effect of bringing the animal from the position of rest on its side with its body in a shallow U to an upright S position, preparatory to swimming. The fact that the first response is always sharply defined makes it the best quantitative measure of the reaction time. However, the first response and the sequence of movements which precede swimming are obviously characteristics of the individual

animals, as are also the time relations between them. Just as some hags have short reaction times and others long ones, so in some the time interval between the first response and the onset of swimming is very brief while in others it is much longer. It may also be relatively constant or highly variable. There does not however appear to be any obvious relation between the reaction time measured to the first response and the interval between the first response and the commencement of swimming. In some animals with consistently short reaction times this interval is relatively long and in others the opposite is true.

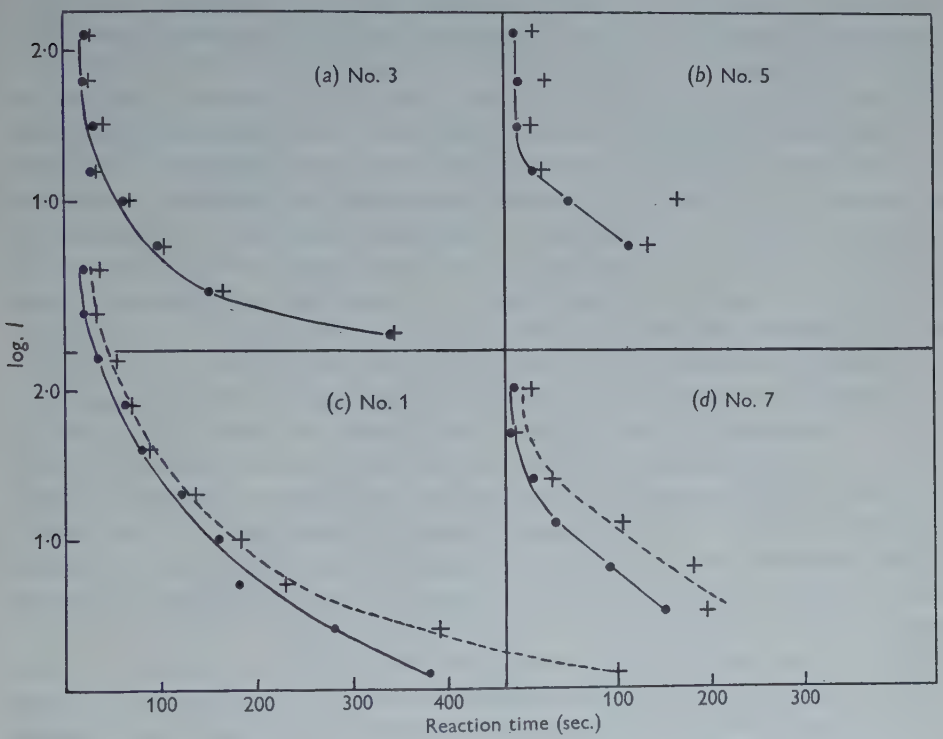

Fig. 2. The relation between the first response and the commencement of swimming of four hags. The curves show the relation between the intensity of the stimulus and the reaction time measured to the first response (dots and continuous line) and the commencement of swimming (crosses and broken line). Light intensity in arbitrary logarithmic units.

One or two examples will illustrate these points. The sequence of responses given by animal no. 3 was almost invariably a sharp twitch of the head and body followed by swimming a second or two later, without any intervening series of movements. This was true at all intensities of stimulus, as illustrated in Fig. 2*a*, which shows the relation between the intensity and the reaction time measured to the first response and to the commencement of swimming for a single series of tests between 0·4 and 50 e.f.c.* For this hag therefore the first response and commencement of swimming were almost simultaneous.

* Brightness values are expressed as equivalent foot candles, e.f.c. 3·426 e.f.c. = 1 candle/square metre.

A similar series of tests on animal no. 5, presented in Fig. 2*b*, shows a different type of relation. The time interval between the first response and swimming varies from 2 to 105 sec., and there is no obvious tendency for it to increase (as does the reaction time) as the intensity of stimulus is decreased. This suggests that photochemical processes play no part in determining this time interval, but only affect reaction times measured to the first response. Other animals, however, did show a tendency for this time interval to increase as the intensity of the stimulus was decreased. This was noticed in several experiments, and will be illustrated here by two examples. The intensity/reaction time data for animal no. 1 between 0·1 and 50 e.f.c. are presented in Fig. 2*c*. At higher intensities the interval varied in an apparently random manner between 6 and 20 sec., the average of seven observations being 15·5 sec., but at the four lowest intensities it increased progressively to the surprisingly long time of 200 sec. at 0·1 e.f.c. In this experiment the animal was illuminated continuously until the first response was seen and the effect is apparent only at levels of illumination approaching the absolute threshold of sensitivity. In the second example, shown in Fig. 2*d*, animal no. 7 was illuminated for 30 sec. at each of six intensities between 2 and 25 e.f.c. Over this range the interval between the first response and the commencement of swimming clearly tended to increase progressively as the intensity was decreased.

The findings illustrated by these last two examples were unexpected and seem to imply that at low intensities of stimulus continuing photochemical processes are in some way concerned not only with the first response but with further processes leading up to the initiation of swimming, i.e. with processes which are usually thought to be entirely secondary. This is another way of stating the point made by Newth & Ross that the light responses of animals with long reaction times, such as *Myxine*, cannot be treated in the classical manner of Hecht as though the reaction time were made up successively of an initial sensitization period followed by a latent period.

It is of course possible, though unlikely, that the intensity of stimulus is related to the commencement of swimming rather than to the first response. It seems better, however, to regard the latter as an alerting movement which can be brought about by a lesser photochemical effect than is required to initiate general locomotory activity. In support of this view it was usual at low intensities of stimulus to find a first response followed by a series of further twitches or local movements, which did not always culminate in swimming. This did not occur at higher intensities. It is difficult to avoid the conclusion that the preliminary local responses and swimming represent respectively two levels of excitation, and that the interval between them represents the time occupied by the build-up of processes to the higher level necessary to initiate swimming. It seems probable too that these processes are thermal and not concerned with the primary photochemical reaction. The position is clearly unsatisfactory as it stands. The data presented however serve to emphasize both the individuality of hags and the fact that the neurological mechanisms, involved are complex compared with those concerned in the light responses of relatively immobile animals, such as *Ciona*, *Mya* and other Invertebrates.

Analysis of reaction time

The relation between the intensity of the stimulus and the reaction time, defined as the period from the commencement of the stimulus to the first response, was studied with single hags exposed to the full radiation of the light source. Measurements of reaction time were made when:

(1) The illumination was constant and the duration of exposure varied.

(2) The duration of exposure was constant and the illumination varied.

(3) Both illumination and the duration of exposure were varied.

(1) Five series of measurements were made, three at 22·6 e.f.c. and two at 63 e.f.c. The exposure times ranged from 1 to 15 sec., the longest being slightly less than the minimum reaction time of the animals. The results are presented in Fig. 3. At 63 e.f.c. the reaction time was almost constant for exposures longer than 4 sec., but increased rapidly for exposures below this value. The reaction time was increased by about sixfold when the exposure was halved from 4 to 2 sec., and abolished when it was further reduced to 1 sec.

The three experiments at 22·6 e.f.c., Fig. 3*b*, show this inverse relation between the reaction time and the period of exposure over the greater part of the range studied. In animal no. 5, for instance, the reaction time was approximately doubled when the exposure was reduced from 7·5 to 5 sec., and doubled again when it was reduced to 2·5 sec. Similarly, the reaction time of animal no. 4 increased by about 10 sec. for each 2 sec. reduction of exposure period below 12 sec.

Approximate values for the sensitization period can be derived from these data. If a hag is illuminated until it gives a response, the reaction time observed is the minimum for that intensity of stimulus. Shorter periods of exposure may not give an increase in the reaction time until a critical value is passed, which is the sensitization period. The latter may therefore be defined as the shortest period of illumination which will give the minimum reaction time at any given intensity. Inspection of Fig. 3 shows that at 22·6 e.f.c. the sensitization period of all three animals is about 10 sec., while at 63 e.f.c. it is about 4 sec. These values can only be considered as approximations, since there is in fact no value of exposure period above which the reaction time can be said to be constant, being affected to some extent by all changes of exposure. This is no doubt due to the fact, which has been pointed out elsewhere, that sensitization and latent periods are really coextensive in time, the former being in *Myxine* a relatively large fraction of the total reaction time.

(2) Three series of measurements were made varying the intensity of stimulus and keeping the period of exposure constant. Periods of 15 and 30 sec. illumination were used, slightly less than the minimum reaction time of these hags. The results presented in Fig. 4 show that the relation between the reaction time and the logarithm of the intensity is linear over a considerable range of illumination. Only the curve for animal no. 5 shows the reaction time approaching a minimum at the higher intensities of stimulus.

(3) By varying both the intensity and duration of the stimulus responses can be obtained over a greater range of illumination than is possible by either of the fore-

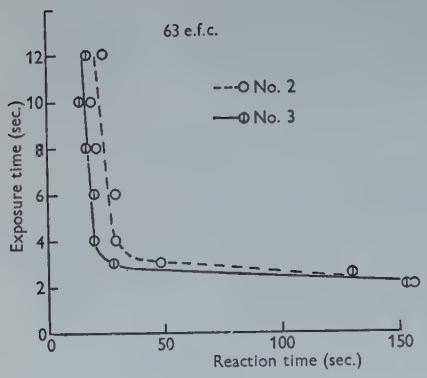

Fig. 3 *a*.

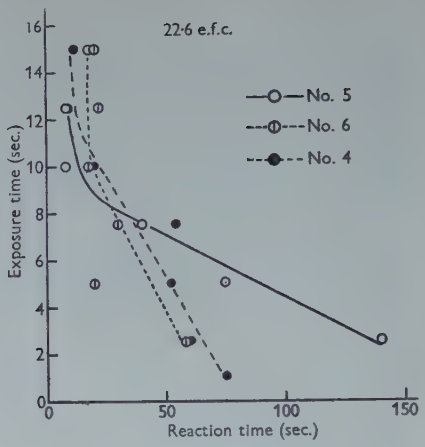

Fig. 3 *b*.

Fig. 3. The relation between stimulus and reaction time for stimuli of constant intensity and varying duration. *a*, at 63 e.f.c., 2 hags; *b*, at 22·6 e.f.c., 3 hags.

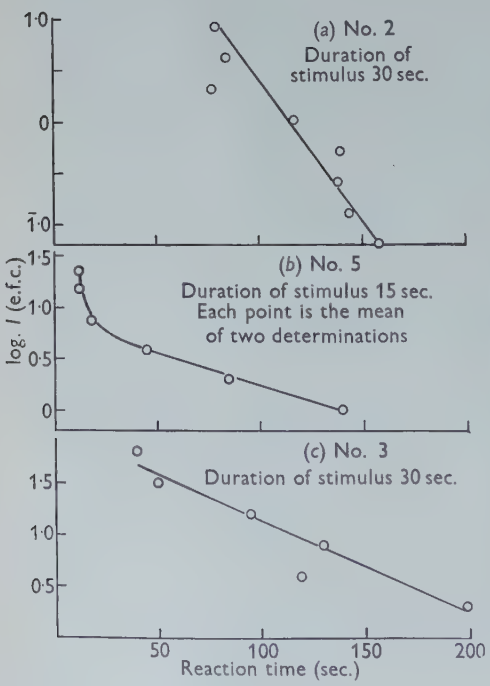

Fig. 4. The relation between stimulus and reaction time for stimuli of fixed duration and varying intensity.

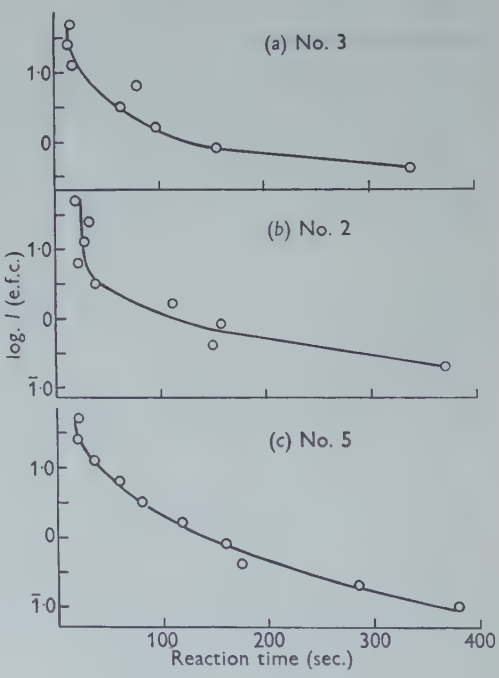

Fig. 5. The relation between stimulus and reaction time for stimuli continued until the first response.

going procedures. In this way we can approach more closely to the minimum threshold of response of each animal and thereby estimate its maximum sensitivity. The procedure was to illuminate the hag continuously until the first response was seen, so that the duration of stimulus was the same as the reaction time in each case. Five such experiments were performed. The results of two of them are presented in Fig. 2*a* and *c*, the other three in Fig. 5. The curves all show the same general characteristics. The reaction times are more or less constant at their minimum value for levels of illumination greater than about 10 e.f.c., and increase rapidly below 1 e.f.c. It is probable that hags could be made to respond to stimuli still weaker than 0·1 e.f.c., which was the lowest level attained (Fig. 5*c*), but the reaction time would be so long that the chance of spontaneous movement intervening would be greatly increased.

Spectral sensitivity

Spectral sensitivity curves were constructed from measurements of the sensitivity of hags in monochromatic light of several different wave-lengths. Spectral bands were isolated by means of the Ilford 'monochromatic' series and other combinations of filters, whose optical properties were known. The relative amount of energy transmitted by each filter in series with the light source was obtained by plotting the product of the energy of the lamp and the transmission of the filter for each 10 mμ of its transmission band. The area enclosed within each curve is proportional to the energy transmitted by that filter. A further correction was made for the quantum

Table 2. *Characteristics of the filters used to isolate spectral bands in combination with* 500 *W. lamp at* 3100° *K.*

Filter (Ilford No.)	Central wave-length (mμ)	Relative transmission
601	430	2·93
602	470	1·36
603	497	1·28
604	519	1·25
807	530	3·85
605	555	1·00
606	578	1·60
808+802	583	14·3
607+802	595	2·15
608±802	650	1·58

effectiveness of the light source and the central wave-length of energy transmitted by each filter, which was in most cases a few mμ towards longer wave-lengths than indicated by the published information on these filters. The characteristics of the filters in combination with the projector lamp used in the Edinburgh experiments are listed in Table 2. The colour temperature of the lamp used at University College was not measured and the energy content of the waveband cannot therefore be calculated with the same accuracy, but the difference between the two series was certainly small and has been neglected in the calculations based on the animal's responses.

Spectral sensitivity was measured in two ways. In the first series of experiments carried out at University College the sensitivity of hags to light of different wave-lengths was calculated from measurements of their dark-adapted thresholds, that is to say by measuring the minimum intensity of light required to elicit a response within arbitrary time limits. An animal was first dark-adapted for about an hour and its threshold determined by subjecting it to a series of stimuli of increasing intensity, with a suitable period of recovery in darkness between each trial, until a response was obtained. This method suffers from the disadvantage that it is difficult to find a satisfactory criterion of a constant threshold response. In these experiments the threshold was defined as the lowest light intensity which gave a reaction time between 90 and 150 sec. in at least two out of three tests. The increment of light intensity between tests was 0·3 logarithmic unit.

Table 3. *Spectral sensitivity of three hags estimated from measurements of the threshold of response*

Wave-length (mμ)	Hag no.					
	4		5		6	
	Log. *I*	Sensitivity (%)	Log. *I*	Sensitivity (%)	Log. *I*	Sensitivity (%)
430	—	—	—	—	1·47	11
470	—	—	1·13	12	0·53	92
497	1·11	24	1·11	39	0·49	100
519	0·49	100	0·19	100	0·50	97
555	0·70	62	0·40	62	1·00	32
583	2·15	2·4	1·61	7	—	—
595	—	—	0·98	16	1·33	15
650	—	—	N.R.*	N.R.*	N.R.*	N.R.*

* N.R. = no response.

A sufficient number of observations was obtained from three animals and the results are presented in Table 3. The energy required to obtain a threshold response as defined above is expressed in arbitrary logarithmic units, and the relative stimulating capacity is the reciprocal of this value. To compare the results from different animals, however, the relative stimulating capacities are expressed in the second column as a percentage of the most effective wave-length. This is the reciprocal of $I\lambda/I_{max.} \times 100$, where $I_{max.}$ is the intensity required to obtain the response at the most effective wave-length and $I\lambda$ the intensity at any other wave-length, λ.

The method adopted in the second series of experiments was similar to that first used by Hecht (1928) with *Pholas*. Instead of measuring the stimulus required to elicit a response in a given time, reaction times for each filter were measured at several intensities of illumination. The results are listed in Table 4. Intensity/reaction time curves for different wave-lengths can be constructed from these data and those for hag no. 3 are shown in Fig. 6. The other two animals gave similar sets of curves.

The relative stimulating capacity of different parts of the spectrum can now be calculated in two ways. The most effective wave-length is that whose curve lies

Table 4. *Reaction times of three hags to stimuli of different wave-length and intensity*

(Intensity of stimulus is recorded in arbitrary logarithmic units of brightness, reaction times in seconds.)

Wave-length (mμ)	Log. *I*	R.T.	Log. *I*	R.T.	Log. *I*	R.T.	Log. *I*	R.T.	Log. *I*	R.T.
Hag no. 1										
430	0·56	292	0·87	235	1·17	160	1·47	150	—	—
470	0·24	200	0·54	178	0·84	110	1·14	125	—	—
497	0·20	220	0·50	170	0·80	120	1·10	100	—	—
519	0·19	310	0·49	232	0·80	185	1·10	125	—	—
555	0·40	230	0·70	138	1·0	105	—	—	—	—
595	1·0	249	1·34	155	—	—	—	—	—	—
Hag no. 2										
430	0·87	263	1·47	185	—	—	—	—	—	—
470	0·54	265	0·84	175	1·14	135	—	—	—	—
497	1·9	258	0·20	205	0·50	220	0·80	80	—	—
519	0·19	325	0·49	260	0·80	144	1·10	150	—	—
555	0·40	280	0·70	212	1·0	130	—	—	—	—
595	N.R.*	N.R.*	—	—	—	—	—	—	—	—
Hag no. 3										
470	0·24	310	0·84	258	1·14	180	1·45	120	—	—
497	0·20	250	0·80	172	1·1	128	—	—	—	—
519	0·19	238	0·49	178	0·80	96	1·1	78	—	—
530	0·39	283	0·68	197	0·98	176	1·29	90	1·58	48
583	1·26	210	1·56	155	1·85	112	2·15	105	—	—

* N.R.=no response.

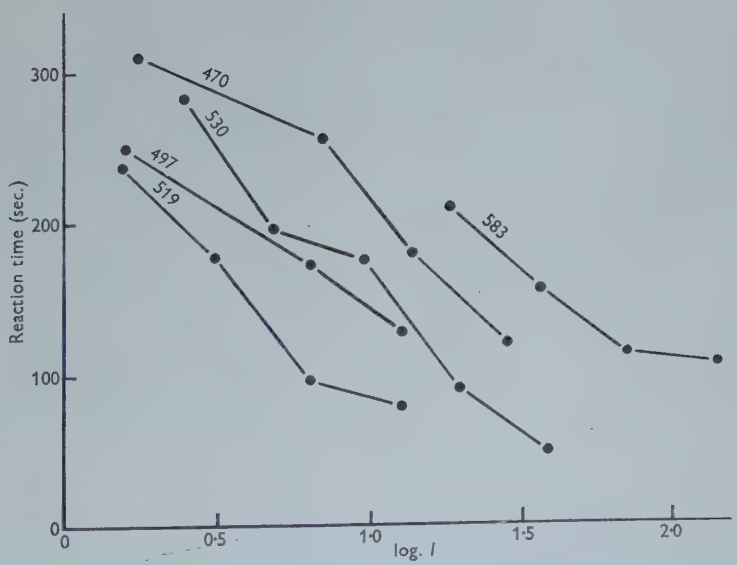

Fig. 6. Intensity/reaction time curves for animal no. 3 at different wave-lengths. The central wave-length in mμ is indicated for each filter beside the appropriate curve.

farthest to the left on the intensity axis (log. *I*), and the relative stimulating capacity of other wave-lengths is proportional to the distance they would have to be displaced along this axis in order to coincide with that for the most effective. Inspection shows, however, that although most of the curves have roughly the same slope, they are not sufficiently parallel to one another to permit them to be compared in this way. The alternative method is to estimate the relative effectiveness of different wave-lengths from the reciprocals of the values of log. *I* for several reaction times. This was done for reaction times of 150, 180, 210 and 240 sec., the results averaged, the reciprocal taken and sensitivity expressed once more as a percentage of the most effective wave-length. The result of this analysis is presented in Table 5.

Table 5. *Spectral sensitivity of three hags estimated from the intensity/reaction time data for different wave-lengths*

(The values for log. *I* are the means of the values for reaction times of 150, 180, 210 and 240 sec.; sensitivity is the reciprocal of log. *I* expressed as a percentage of the value for the most effective wave-length.)

Wave-length (mμ)	Hag no.					
	1		2		3	
	Log. *I*	Sensitivity (%)	Log. *I*	Sensitivity (%)	Log. *I*	Sensitivity (%)
430	1·08	41	0·79	21	—	—
470	0·78	58	0·80	34	0·89	38
497	0·45	100	0·27	100	0·61	70
519	0·67	68	0·61	45	0·43	100
530	—	—	—	—	0·79	55
555	0·58	77	0·68	39	—	—
583	—	—	—	—	0·72	31
595	1·51	29	N.R.*	N.R.*	—	—

* N.R. = no response.

The spectral sensitivies of the six hags vary considerably and illustrate one of the disadvantages of filters for this type of work. Since sensitivity has conventionally to be expressed as 100% at a given wave-length, a small difference in the observations may shift the maximum from one filter to another. Curves for single animals are therefore of little value, but considering the observations as a whole we may reasonably draw the following conclusions concerning *Myxine*'s spectral sensitivity:

(1) The most effective wave-length is between 500 and 520 mμ, since the most effective filter was either no. 603 or no. 604 (three hags in each case).

(2) Sensitivity decreases rapidly towards wave-lengths longer than the maximum. At 583 and 595 mμ about 10 times as much energy was required to produce the same effect as at the maximum. *Myxine* is virtually insensitive to wave-lengths longer than about 600 mμ.

(3) Sensitivity appears to decline less rapidly towards shorter wave-lengths, but observations at this end of the spectrum are too few to permit much freedom of speculation. At 470 mμ sensitivity seems to be about half what it is at the maximum.

Fig. 7, which represents the arithmetical mean of the spectral sensitivity curves of the six hags, may be considered as a summary of these conclusions. It is probably not greatly different from the true spectral sensitivity of *Myxine*.

Analysis of Vitamin A and carotenoids

Vitamin A is a component of all known photosensitive systems of higher animals, and other carotenoids are commonly associated with them. As no information seemed to exist on the occurrence and distribution of these substances in *Myxine*, two live hags weighing about 100 g. each were sent for analysis to Dr T. W. Goodwin of the University of Liverpool. He reported in a private communication (1952) that their combined livers contained 45 μg. and the rest of their bodies 110 μg. of vitamin A_1. They contained no other carotenoids and no vitamin A_2. If we

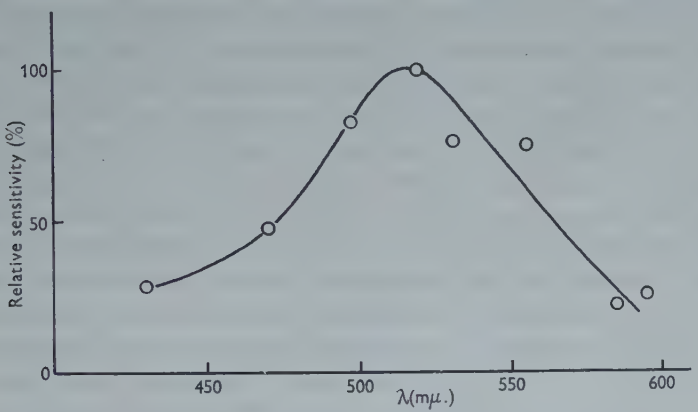

Fig. 7. Smoothed spectral sensitivity curve for six hags. Each point represents the mean value for all animals, calculated from the data presented in tables 3 and 5, and expressed as a percentage of the value for the most effective wave-length.

assume that the vitamin A was equally distributed between the two fish, each would have contained about 70–80/μg., of which about 20 μg. was in the liver. These quantities are small compared with the vitamin A content of many other fish, but it must be remembered that the hags had been kept in the laboratory for about 4 months, during which time they took no food. Although they appeared to be perfectly healthy and active up to the time they were killed, we might expect their reserves of the vitamin to have declined during this period. The normal vitamin A content of well-fed hags may therefore be much greater than these figures suggest.

DISCUSSION

The significance of the light reaction

Many of the anatomical peculiarities of hags, such as their lack of functional eyes and almost complete lack of integumentary pigment, suggest that they normally spend their lives in darkness, possibly in a marine equivalent of the cavernicolous environment. A general sensitivity to light such that illumination of almost any

part of the body surface may result in a motor response is a common feature of animals which inhabit these places, and has been studied by Hawes (1946) on the blind Urodele *Proteus*. To such animals a light sense of some kind is not, as Hawes suggested, a functionless evolutionary survival but a practical necessity and the only insurance they possess against wandering into illuminated places where they would be without protection from predators with image-forming eyes and normal vision. Newth & Ross have discussed this matter briefly, and suggest that the main role of *Myxine*'s light sense may be to keep it buried during daylight hours or to prevent it from migrating into shallow water. It seems worth while to pursue this idea a little further. If the hag's light sense plays any part in its life we might expect it to be related quantitatively to the level of illumination likely to be encountered at the depths where they are found in the sea. So far as is known hags are inhabitants of the continental shelf and appear to be confined to muddy bottoms. Gustafson (1935), who studied their habits in aquaria, found that they remain buried during the daytime but swim about freely and feed at night. If they behave in the same way in their natural habitat it follows that they must at least be able to distinguish night from day. Although they are taken sometimes from considerable depths, many known hag grounds are in quite shallow water between 10 and 50 m., where the illumination from the sea's surface during the day is by no means negligible. The illumination at any given depth is of course affected by many variables, such as the turbidity of the water, the disturbance of the sea's surface due to wave action, the altitude of the sun, latitude, amount of cloud and so on; but we need concern ourselves only with the amount of light energy which penetrates the surface and the fraction of it which is absorbed by each successive layer of water of unit thickness, a function usually expressed as the absorption or extinction coefficient per metre. The value of 120,000 lux, which is equivalent to 11,200 f.c. has been given by Sverdrup, Johnson & Fleming (1942) for the radiation penetrating the surface with a clear sky and sun at zenith. Assuming an overall absorption coefficient of 0·29 per metre for coastal water of average turbidity, the illumination at 30 m. depth will be 0·22 f.c. In clearer oceanic waters the same illumination might be encountered at 4 to 5 times this depth. Inspection of the intensity/reaction time curves (Figs. 2–5) shows that hags were activated within 3–4 min. by this intensity, which is therefore clearly within their effective range of perception.

Although these estimates are of necessity based upon arbitrary values for the amount of radiation and the turbidity of the sea, they certainly support the view that the hag's light sense is sufficiently acute to enable it to respond to the degree of illumination likely on the sea-bed in daytime at the depths in which they are commonly found. If it serves no other purpose this reaction has probably a high selective value, since it will have the effect of keeping the hag on the move until it finds itself on a suitable substrate into which it can burrow, or until it has found its way into deeper water.

Spectral sensitivity and the photochemical system

Although the data leave much to be desired, it is clear that the spectral sensitivity of the hag in its general features is similar to that of other marine animals, such as *Ciona*, *Pholas* and *Mya*, studied many years ago by Hecht. The most effective wave-length in every case is in the blue-green or green region of the spectrum: and sensitivity decreases towards both longer and shorter wave-lengths. It is axiomatic that the relative stimulating capacity of different parts of the spectrum is determined primarily by the energy absorbed by the photosensitive pigment responsible for the reaction, and the spectral sensitivity curve may therefore be regarded as an indirectly determined absorption spectrum of that pigment. Action spectra of this kind provide, in fact, the only information we possess on the photochemical systems of most animals, since our direct knowledge of the chemistry of visual pigments is confined to those of the image-forming eyes of Vertebrates, Cephalopods and some Arthropods. It is thought that in other animals the amounts of photopigment are too small or too diffusely scattered throughout the body to be detected by any of the techniques at present available. We can therefore only speculate on their photochemical systems by comparing the action spectra with those of animals possessing eyes and by analogy with those visual pigments whose chemical properties are known.

The spectral sensitivities of *Mya*, *Ciona*, *Pholas*, the ammocoete larva of *Lampetra* and the hag, differ from one another most obviously in the position of the maximum or most effective wave-length, which ranges from about 500 mμ in *Mya* to about 550 mμ in *Pholas*. This suggests that the photosensitive substances differ in detail, but are probably all of the same general type. Their relationship may well be similar to that of the different types of rhodopsin or visual purple, the best known photopigment of Vertebrate eyes. At one time rhodopsin was thought to be a single substance with well-defined physical and chemical properties, which were identical in all animals possessing it. The variations observed were thought to be due to impurities or to differences in the methods of preparation and analysis. It is now clear, however, that the position of the maximum of the absorption spectra of rhodopsin from different animals varies to some extent, and in Wald's (1953) words 'the term rhodopsin...therefore, like haemoglobin, designates a family of closely related substances'. This is due to the fact that the protein, or opsin, part of the photopigment affects the position of the absorption maximum to some extent and is a characteristic of the species. Among vertebrates this effect is rather small, only accounting for a range of about 5 mμ, but is greater in the case of rhodopsin from Cephalopods, and it seems reasonable to expect that the variation due to different proteins will be larger between animals that are more distantly related phylogenetically.

Most of the larger differences in absorption spectra are due to different prosthetic groups, and it is usual then to assign different names to the resulting pigments. The best known example of this is the case of rhodopsin and porphyropsin, which differ in the type of vitamin A in the chromophore, and whose absorption maxima lie

at about 500 and 522 mμ respectively. The absorption spectrum can also be affected by the various stereo-isomeric forms of vitamin A, or more correctly the corresponding vitamin A aldehydes, or retinenes. It seems too that photosensitive pigments, which differ chemically only in respect of their proteins but possess markedly different absorption spectra, may exist together in the eye of the same animal. Wald, Brown & Smith (1952) have recently found this to be the case in the chicken, the eye of which contains rhodopsin and another pigment, iodopsin, whose maximum lies at 562 mμ. The chromophore group of both pigments is the same, neo-retinene *b*, but the proteins are different.

It is clear, therefore, that photosensitive pigments with different spectral absorptions can be formed in a number of ways, and the range of variation of the maxima seems adequate to account for the differences between the spectral sensitivities of the animals with which we are concerned. It is indeed probable that the photoreceptor systems of all higher animals are constructed on the same pattern of a vitamin A-containing chromophore united with a specific protein.

This possibility opens up an interesting field of speculation on the evolution of photoreceptor systems. Their uniformity in all higher animals suggests that the basic chemical plan was fixed once and for all at a very remote time, certainly before the Vertebrates appeared on the scene, and that forces exist which have tended to keep them constant however much the animals may have evolved in other respects. The explanation may well lie in the spectral transmission of water, which is extraordinarily similar to the absorption spectrum of rhodopsin and to the relative sensitivity of various animals. Pure water is most transparent to wave-lengths between 400 and 600 mμ, while longer wave-lengths are progressively more strongly absorbed. Natural sea and fresh waters are more opaque, due principally to scattering of the light by small particles in suspension, and also deviate to some extent from pure water in their spectral transmission. Thus the maximum penetration of clear ocean water by light is at about 480 mμ in the blue region of the spectrum, while coastal waters are most transparent to green of about 530 mμ or even longer wave-lengths. In recent years several excellent series of measurements have been published of the transparency of sea water to light of different wave-lengths. The data are usually presented as extinction or absorption coefficients, but are more revealing for our purposes when the energy at each wave-length is expressed as a fraction of the energy at the wave-length to which the water is most transparent. The spectral distribution of energy at any depth can then be compared directly with the sensitivity curves of animals, which may be regarded as representing percentage absorption curves of a particular concentration of photosensitive pigment. Some of Utterback's (1936) figures for the average of several coastal and oceanic waters are expressed in this form in Fig. 8. They show clearly that the most effective wave-lengths in terms of their stimulating capacity are just those to which sea water is most transparent and vice versa. To emphasize this the values have been calculated for arbitrary depths of water so as to yield curves whose slope corresponds fairly closely with known spectral sensitivity curves. At greater depths the curves would be narrower and more sharply peaked, for shallower

water correspondingly broader, without however altering the position of the maxima.

The variation in spectral transmission of different types of sea water is certainly as great as the differences in spectral sensitivity of those animals which have been studied so far, and it is tempting to speculate that there may be a correlation between the ecology of an animal and its spectral sensitivity. If within the limits imposed by the general chemical properties of the system, selection can take place for a pigment of maximum effectiveness in any given environment, we might expect to find pelagic species from oceanic waters with maxima at rather shorter wave-lengths than the inhabitants of coastal seas. So far as I am aware, however, there is no experimental evidence that this is so. Bayliss, Lythgoe & Tansley (1936) attempted

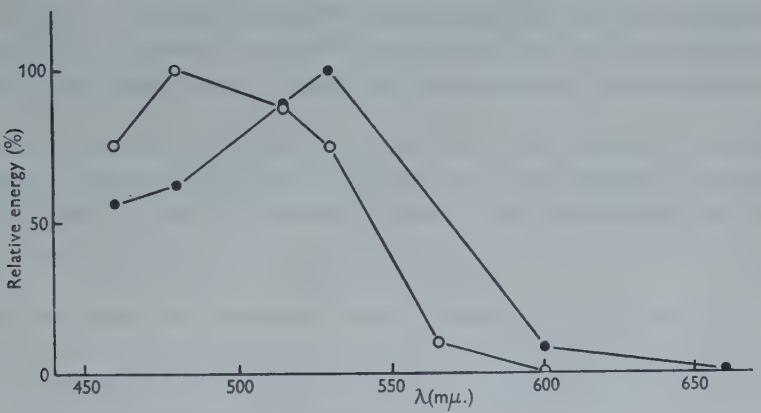

Fig. 8. The relative energy content of light of different wave-lengths penetrating oceanic and coastal waters. Open circles indicate average of ocean waters at 100 m.; filled circles average of coastal waters at 20 m. Data recalculated from Utterback (1936).

an investigation on these lines and found photosensitive pigments with maxima ranging from 505 to 545 mμ in the eyes of twelve species of fish. None of them were oceanic forms however, and new doubt has been thrown recently on the accuracy of their measurements by Kampa (1953), who found only rhodopsin in three of the species which were thought to possess pigments with the maximum at longer wave-lengths.

A further consequence of this hypothesis is that it can provide a reasonable explanation for the spectral properties of rhodopsin and the scotopic visibility curve of terrestrial Vertebrates, of which one of the most puzzling features is the virtual insensitivity to wave-lengths longer than about 600 mμ. There seems no obvious reason why the visual sense of a terrestrial animal should be limited in this way, but it is clearly adapted to the aquatic environment in which it was first evolved. On this ground alone there is good reason to consider rhodopsin and its variants as the 'primitive' visual pigment of all higher animals.

SUMMARY

1. The response of the hag to light consists of one or more local movements followed after a further interval by general locomotory activity. The first local movement has been used as a measure of the reaction time.

2. The reaction time is inversely proportional to the intensity of the stimulus at illuminations less than about 10 e.f.c. At higher levels of illumination it attains a constant minimum value. Hags respond to intensities at least as low as 0·1 e.f.c. but only after several minutes illumination.

3. Estimates of the penetration of light through sea water suggest that the hag's light sense is of functional value.

4. The spectral sensitivity maximum lies between 500 and 520 mμ. Hags are virtually insensitive to wave-lengths longer than about 600 mμ.

5. The significance of the spectral sensitivity is discussed in relation to the spectral transmission of sea water and the evolution of photosensitive systems.

I wish to thank Messrs Newth and Ross for allowing me to work with their hags and for their collaboration in this work; also Prof. P. B. Medawar, F.R.S., and members of the staff of the Zoology Department at University College.

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AN INVESTIGATION OF THE 'CHRONOMETER' FACTOR IN BIRD NAVIGATION

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INTRODUCTION

In previous papers (Matthews, 1951*a*, *b*, 1952, 1953*a*, *b*, *c*) the existence of a true, bico-ordinate navigational ability was demonstrated in three very different species of birds, Homing Pigeons, Lesser Blackbacked Gulls and Manx Shearwaters. In each case the sun was shown to play an essential part in such navigation, which broke down with overcast conditions. A theory of complete sun navigation proposed was found to be theoretically plausible. Experimental evidence was obtained that pigeons were detecting latitude displacement from differences in the noon altitude of the sun at home and at the release point. A start was made with the investigation of the second part of the hypothesis, that longitude displacement was detected by comparing home time (provided by an internal 'chronometer') with local time at release (estimated from the sun's position on its arc with reference to the highest point of that arc). Exposure of pigeons to irregular light/dark sequences, such as might disrupt the working of such a 'chronometer', resulted in a breakdown of orientation. An attempt to obtain reorientation in longitude with shearwaters by exposure to regular, out of phase, light/dark sequences gave suggestive results. The present paper describes further experiments on these lines.

Manx Shearwaters (*Procellaria puffinus puffinus*, Brunn.) were taken from the colony on Skokholm Bird Observatory, Pembrokeshire. The technique of obtaining and handling these birds has been described before (Matthews, 1953*c*). Pigeons were reared at the Ornithological Field Station, Madingley.

I. ATTEMPTS TO IMPOSE A DIRECT SHIFT ON THE 'CHRONOMETER'

Method

From the previous experiments it seemed probable that the normal light/dark sequence acted as a pacemaker for the birds' 'chronometers'. It was therefore proposed to attempt to advance or retard the 'chronometers' by arranging an artificial day beginning and ending a number of hours earlier or later than normal. If displacement in longitude is indeed detected by differences between home ('chronometer') time and local time at release, then the birds with altered 'chronometers' should be orientated falsely. A complicating factor which must be taken into consideration is that the advanced or retarded day would be equivalent to that obtaining a long way to the east or west of home. It is therefore possible that the

birds might 'consider' that the transfer to the new day conditions was brought about by their having been displaced in longitude, and remember this when released. This might occur independently of, or together with, the effects on the 'chronometers'. Thus for birds subjected to an advanced day and then released say 60 miles east of home we have the possibilities shown in Table 1.

In case A, when the treatment has been wholly ineffectual, the experimental birds will show no difference from the untreated controls. Not much difference could be expected in case B, although the reinforced westward tendency might be apparent. In case D the effects might cancel out, giving no orientation in longitude, or one or other of the indications might be 'preferred'. Only in case C could we expect a radically different initial orientation between experimentals and controls.

It is thus clearly most important that the change in the light/dark sequence should not be interpreted as being due to displacement in longitude. This could best be done by carrying out the treatment in familiar surroundings at home, or in

Table 1

	Artificial day has effect of		Sun at release in relation to 'chronometer' time	Expected flight direction
	Advancing 'chronometer'	Being interpreted as shift to east		
A	No	No	Slightly in advance	West
B	No	Yes		West
C	Yes	No	Far behind	East
D	Yes	Yes		Indeterminate

conditions that closely simulate these. Further, while the change in 'chronometer' time must be sufficient to offset the actual displacement to the release point (otherwise birds in case C will still fly west), it should not be so large that there is a possibility of the orientation mechanism breaking down in such 'impossible' conditions. Even if longitude displacement could not then be detected it is possible that latitude displacement could still be detected, since the sun's noon altitude could, theoretically, be estimated without a chronometer, from the observed sun arc. Then if the release point differed in latitude from home, orientation north or south as appropriate would be expected (this applies in case D also).

It was not possible to treat shearwaters on Skokholm owing to the lack of a soundproof room which would be needed to exclude the noise of the other shearwaters returning at night. The birds selected for the experiment had not been used in tests before, so the disturbance of removing them to the mainland would have no meaning in terms of displacement. Any elaborate kinaesthetic sense recording spatial displacement has been disproved (Matthews, 1951*b*). The journey to Cambridge, once started, might therefore reasonably be completed. To try and create a 'homelike' atmosphere in the room there, the birds were provided with:

(1) a confined space, similar to the nesting chamber,

(2) nesting material,

(3) an imitation egg (such had proved readily acceptable in the burrow),

(4) a recording of the nocturnal uproar made when the birds are returning to their burrows. As suggested previously this nightly noise may be an important pace-maker for the birds' 'chronometers' in addition to the light/dark rhythm.

The alternation between artificial day and night was arranged by raising or lowering the illumination (two 100 W. bulbs in an 8 ft. cube) by four stages over half an hour. The length of the night was that appropriate to the season, and at the appropriate time the record of calling was played, the natural sequence being reproduced by increasing the amount and loudness of calling to a crescendo, and then making it die away more slowly, the whole process taking 2 hr.

With these precautions it was hoped that if the shearwaters responded at all to the artificial day it would be by an adjustment of their 'chronometers' to the new time. The time shift imposed was arbitrarily taken at 3 hr., and was the same for all experiments described in this paper. This was quite sufficient to mask the time shift due to the actual change in longitude (about 20 min.), but not, it was hoped, so large as to disrupt the 'chronometer' mechanism. The artificial day was in advance of normal. A similar number of shearwaters were kept in their boxes (without an egg) in a separate wing of the building as controls. Normal daylight coming through frosted windows facing north was the only illumination provided in this case. The period for which the treatment could be continued was limited to that for which the birds could be restrained without being weakened. They would not take food, but would swallow water jetted on to their beaks. Although they can remain continuously on the nest for up to 16 days (Matthews, 1954), the average incubation shift is about 5 days. In view of this, and of previous experience, a limit of 4 days and nights in the boxes was imposed. This was very short for the purpose of the experiment and also meant that the birds had to be released at the end of this time whatever the weather conditions might be.

There was no such urgent time limit with pigeons since they could be kept under fairly normal conditions, with room to exercise. Again the experimental birds could not be kept at the loft site owing to the lack of sufficiently spacious sound-proof accommodation. They were therefore transported the short distance to the laboratory ($3\frac{1}{2}$ miles) in uncovered baskets, along a road with which they might well have become familiar in previous moves to Cambridge prior to long journeys. They were kept in the same lightproof room previously used for the shearwaters, of about 500 cu.ft. capacity, equipped with a sufficient number of the usual box perches on the walls. The artificial day was again 3 hr. in advance of normal. Temperature was not controlled, but the previous experience in this room (Matthews, 1953*a*) had shown that the heat of the bulbs was sufficient to ensure that it rose to a maximum during the artificial daytime. Ventilation was ensured by a masked fan system. The control birds were kept in the loft with a similar space at their disposal, but with full view of the sun and sky. Both groups were fed twice a day at constant times, those for the controls being normal, those for the experimentals being advanced accordingly.

The shearwaters used in the three experiments to be described (Cambridge J, K, L) were completely untried birds so it was reasonable to expect the two

samples to be equally matched if there were any variations in homing ability. The experiment with pigeons (T. 38) was carried out with thirty-eight birds that had been bred and used experimentally in previous years and retained for breeding purposes. Their experience is indicated in Fig. 2. Nine birds reared and used in 1951 and seventeen reared and used in 1952 had received their training from the west of the loft, followed by a single critical release to the south. Nine reared in 1950 had been trained from the north and given critical releases to the west and south. Three from 1952 had not been trained. Sixteen of the 1951–2 birds had had the experience of being shut off from sun and sky in the altitude/latitude experiments (Matthews, 1953*a*), but no attempt had been made to upset their 'chronometers' on that occasion. The birds had been mated up on their field performance, like with like, and alternate pairs in order of merit were allotted to the experimental and control groups. With a few adjustments each group contained like numbers of north-trained birds, of birds with experimental experience, of untrained birds and of males and females. These birds had been allowed to rear two pairs of young in 1953, the process being complete by the end of May. They were then allowed to remain mated and in possession of their nesting boxes, to keep them in breeding condition and delay the onset of the moult. In the week before the experiment began they were given a short 'refresher' course of short-distance releases at 5, 10 and 25 miles south.

The shearwaters were taken to the release point in their (covered) boxes by day, that is, after it was light both inside and outside the experimental room. The pigeons were placed in covered baskets and loaded into the lightproofed van when it was dark both inside and outside the room, around 23.00 hr. The journey to the release point was thus made through the night. The interior of the van was dimly lit at local sunrise to emphasize the time lag behind the conditions in the experimental room. Thus no bird had experience of the outside conditions from the beginning of the experimental treatment until immediately before its release.

Birds were released singly, being tossed straight up into the air, the liberator facing in a different direction each time. A bird was followed in 16×40 binoculars until lost from sight, when its bearing and the time lapse from release were noted. Only then was the next bird released. Two control releases would be followed by two experimentals to lessen further the chances of birds from the two groups joining up out of sight of the release point. The pigeons, as in previous experiments, were released in flat open country carefully selected for good all-round views. The shearwaters were also released from the ground, in fen country a few miles to the north of Cambridge. It had been found that in previous releases from the 180 ft. tower of the University Library the advantages of the height were offset by the difficulty of following birds that flew below the level of the tower. In a ground release the birds are nearly always silhouetted against the sky and much easier to follow for a longer period. Neither species flew very high, the usual range being 100–200 ft. A few very low flyers lost from sight in less than a minute, and so giving unsatisfactory orientation evidence, are omitted from the scatter diagrams.

The co-ordinates of the theoretical release point in relation to home, as they would appear to a bird using sun navigation and taken in completely by the experimental treatment, are:

Latitude: the actual shift in latitude to the real release point, plus or minus the equivalent in latitude of the change in the sun's declination in the period for which the birds were out of sight of sun and sky.

Longitude: the equivalent of 3 hr. time shift in the appropriate latitude, less the actual shift in longitude to the real release point.

The initial direction (A) in which the birds should then start was calculated from the formulae

$$\text{hav } p = \text{hav } P \times \sin a \times \sin b + \text{hav } (a \sim b)$$

$$\text{hav } A = \{\text{hav } a - \text{hav } (b \sim p)\} \operatorname{cosec} b \times \operatorname{cosec} p,$$

where a = colat of home,

b = colat of 'release point',

P = difference in longitude between 'release point' and home.

The initial scatter diagrams can be examined by 't' or χ^2 tests. It was shown (Matthews, 1953*a*) that the former is useful for comparing well-balanced scatters about a single bearing. But its usefulness breaks down when a distribution is heavily skew about the home bearing, or, more particularly, when the distribution may be a compound one, with two opposed bearings about which the birds may be scattering. In these circumstances, which obtain throughout this paper, resort must be made to the χ^2 test, the division of the diagram adopted being such that a substantial number of points could be expected in the sectors. A simple bisection has been used throughout. To reduce the occurrence of statistical insertions it may be assumed that a given result does not reach the $P = 0{\cdot}05$ level of significance if no statistical evidence to the contrary is offered.

Results

In the first experiment with shearwaters (Cambridge J, 28 May 1953) fourteen experimental birds and eleven controls were released between 05.36 and 07.23 hr. (G.M.T. used throughout) after four nights of the advanced time treatment. Although sun conditions were good ($\frac{4}{10}$th Cu) there was a strong wind (force 4), though blowing from a neutral direction, north-north-west. Home was 235 miles away on a bearing 259°; the theoretical false bearing being 073°. It will be clear from the scatter diagram (Fig. 1*a*) that the wind had a strong deflecting effect on these birds which were not in the best of condition after being in their boxes for 4 days. But it is also clear that there is no difference between the scatters for experimentals and controls, no more tendency for the former to be lost from sight in an easterly direction. The treatment had failed to have the expected effect. The time lapse from release to vanishing was similiar in both groups, 2·9 and 2·6 min., respectively. The experimentals actually gave the faster returns to Skokholm (Table 2).

In view of the disturbing effect of the wind in this experiment, it was repeated in the hope of obtaining better weather conditions. On the next occasion (Cambridge K, 24 June 1953) sixteen birds of each group were released after the same treatment

Table 2

	Returned on nights			Missing	Total
	1–4	5–10	Later		
Experimentals	6	7	1	—	14
Controls	3	3	3	2	11

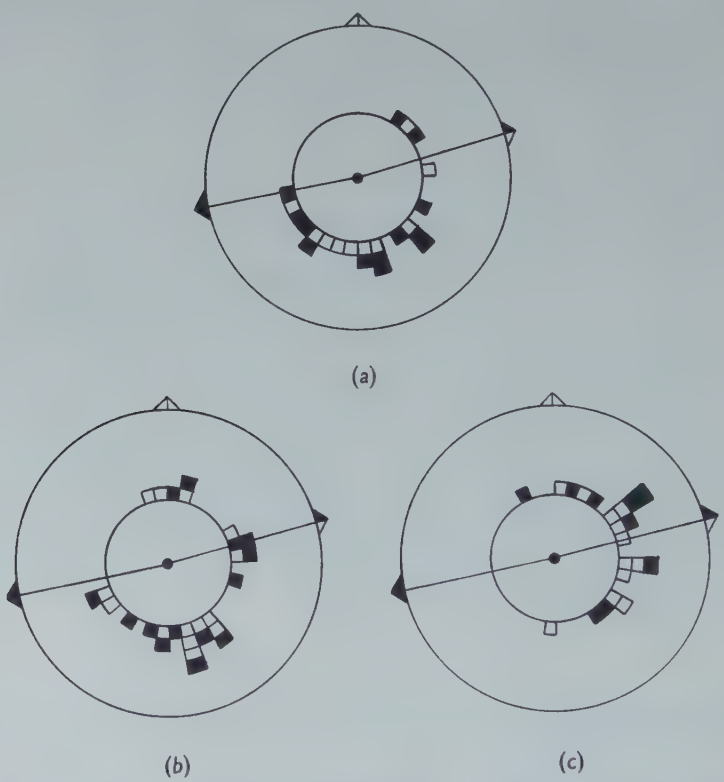

Fig. 1. Initial orientation of shearwaters after attempts to impose a direct 'chronometer' shift. (*a*) Cambridge J, (*b*) Cambridge K, (*c*) Cambridge L. Experimental birds in black, controls in white.

General note on orientation diagrams:

Vanishing points grouped in 10° sectors. Solid arrow, true home bearing; half-blocked arrow, predicted false bearing; open arrow head, true north.

as before, 07.25 to 11.46. Unfortunately, the sky was completely overcast and visibility poor. The initial scatter (Fig. 1*b*) was what previous experience would lead us to expect, no homeward orientation, a generally random dispersal with a downwind tendency even though the wind was light (North force 2) and would have no effect in sunny conditions. There are no differences between experimentals

and controls as regards orientation or time in sight (2·5 *v.* 2·6 min.). Again in line with previous results the returns were slower than in the earlier sunny release, but with no difference between the groups (Table 3).

One further attempt was made (Cambridge L, 8 July 1953) with ten experimentals and thirteen controls released between 04.50 and 06.59 hr. Sun conditions were good, only high thin cloud 2–5/10th, but once more the wind was strong (force 4–5) and, worse, blowing from due west. Experimentals and controls alike were driven downwind (Fig. 1*c*) and lost from sight in times that do not differ significantly, 3·2 and 2·2 min. No check for returns was made, as the season for homing work with this bird on Skokholm had come to an end.

Including the 1952 tests Cambridge F and I, five experimental releases had now been made to test the 'chronometer' factor in shearwaters, involving 123 birds. Not once had wholly suitable weather conditions been encountered and the results must remain inconclusive. But as far as they go, particularly in Cambridge J, they do indicate that four nights of the treatment were quite ineffectual at upsetting longitude orientation. A parallel experiment, but with longer treatment, was now carried out with the homing pigeons.

Table 3

	Returned on nights			Missing	Total
	1–4	5–10	Later		
Experimentals	1	12	—	3	16
Controls	4	11	—	1	16

The treatment of the experimentals, aimed at advancing the 'chronometer' by 3 hr., was continued for ten complete days. By the third and fourth days it looked, from casual observation, as if the birds had settled down to the artificial day. For instance, they appeared to be roosting well in advance of the normal time. But too much stress should not be laid on this, since in normal circumstances some birds will be sleeping while others are active. In particular, such activity changes cannot be taken as evidence that the 'chronometer' has been changed. Stein (1951) has shown that long after activity rhythms of passerines had broken down under continuous lighting, the birds were still indicating the time at which they had been trained to expect food. On 11 August 1953 the birds were released between 06.00 and 09.39 hr. in ideal weather conditions, cloudless sky and a slight breeze, east-south-east, force 0–1 (T.38). At the release point (see Fig. 2) home was 56 miles away on a bearing of 252°, the false bearing being 079°. This was the most easterly point available which would still be a good distance (20 miles) from the coast. Other releases (T.3, 4, 9, 13, 14, 21, 36, 41, 42) have been made rather closer to a coast without the orientation being biased. The centre of a deserted airfield provided an ideal release site in otherwise rather unsuitable country.

Although neither experimentals nor controls gave particularly closely fanned scatters (Fig. 3) they were both non-random and orientated towards home (χ^2,

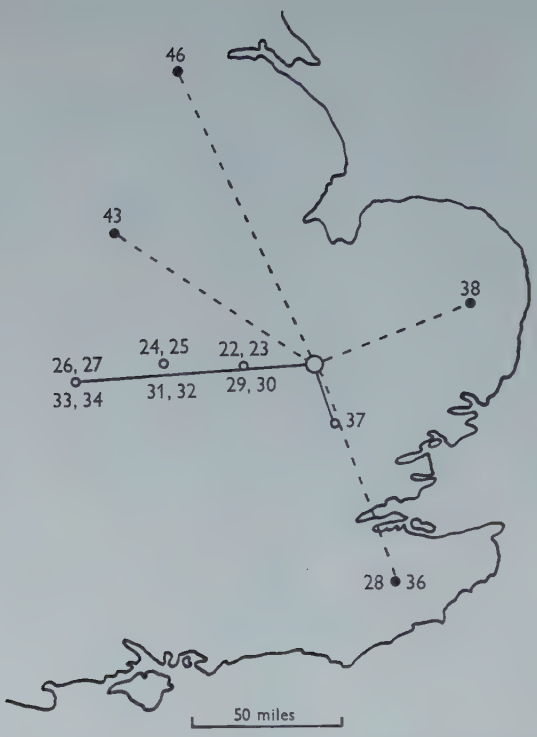

Fig. 2. Release points of adult pigeons. Solid lines and open circles, training releases; dotted lines and solid circles, test releases. 1952, T.29–36; 1951, T.22–28; 1950 not shown for clarity, training line approximating to that of T.46 with test releases west-south-west and south, T.10–21. All releases at T.37 in 1953. Only releases at 25 miles or further from home are shown.

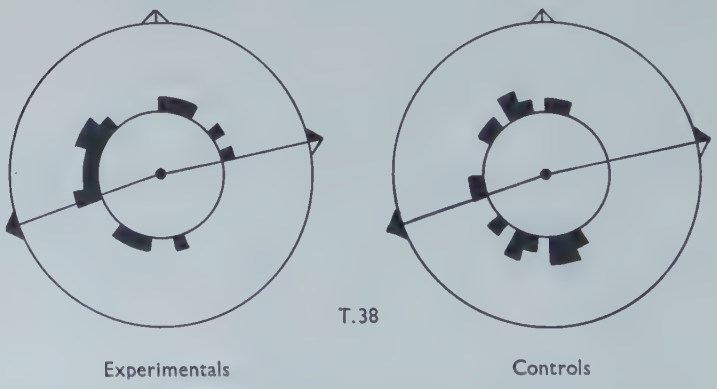

Fig. 3. Initial orientation of adult pigeons after attempt to impose a direct 'chronometer' shift. T.38.

P being respectively 4·26, 0·04 and 5·55, 0·02 when the scatter is bisected at right angles to the home bearing). If anything, the experimentals show a higher proportion of birds closer to the home bearing (13 *v.* 7 within 60°). The birds were in sight for similar times, averaging 3·7 and 4·0 min. Once again the time-shifting treatment had failed to affect initial orientation, confirming the tentative conclusions reached with the shearwaters. The experimental pigeons also homed rather faster (Table 4).

Table 4

	Returned on days				Missing	Total
	1	2	3 and 4	Later		
Experimentals	7	1	6	4	1	19
Controls	2	7	4	4	2	19

The results thus far might mean that 'chronometers' are not concerned in longitude navigation, but a great deal of negative evidence would be necessary for this conclusion to be accepted. It was also possible that the experimental treatment was insufficient to upset the 'chronometers', and more drastic treatment was therefore decided upon. There is the additional possibility that the shift in the artificial day was being interpreted as due to a shift in longitude, a long way to the east (case B, p. 40). Both shearwaters and pigeons released in sunny conditions gave rather faster returns by the experimentals. This would be expected if on release they had a stronger conviction than the controls (with only a small time shift to estimate and interpret) that they must fly west. The somewhat better orientation of the experimental pigeons also lends support for this view. More definite evidence could be obtained by releasing birds treated in this manner, in a different direction, say to the south. They should then still fly west while controls should go north. This would be strong indirect proof that longitude determination had a chronometer basis of the kind proposed, but it would be less satisfactory than the type of re-orientation which would be expected from an alteration in the 'chronometers' themselves.

II. ATTEMPTS TO DISRUPT AND THEN IMPOSE A SHIFT ON THE 'CHRONOMETER'

Method

The experiment (T.35, Matthews, 1953*a*) of subjecting pigeons to an irregular light/dark rhythm had resulted in a random initial scatter. It was decided to use this technique again to throw the 'chronometers' out of 'gear', when it should be easier to reset them to a new time by imposing a regular light/dark rhythm. Such a combined technique had another advantage in that the birds would settle down in the lightproof room while subjected to a most irregular day which could not possibly be interpreted in terms of a change of latitude. As further emphasis of the nearness of the lightproof room to home, the experimental birds were not only

brought to the laboratory in open baskets, but left in them on the flat roof, with a view towards the home surroundings, for 4 hr. on a sunny afternoon. They were then taken straight down to the experimental room. The control birds were given a similar exposure in the afternoon before transport to the release point. In other respects the technical arrangements were as before.

Four experiments were made, T.43-46, and as similar results were obtained, they will be considered together. In T.43 the 34 old birds that had then returned from T.38 were used as a try-out of the new method. Since the experimentals had given the better performances in that test, any effect would have to be manifest in them to be convincing, so they were again used as experimentals and the former controls again as controls. In T.44 young birds were used that had been hatched between 16 March and 16 May in the present (1953) season. Their training was rather different from that given in previous years. It had been shown (Matthews, 1953*b*) that training did not greatly improve initial orientation from a novel direction. But it did considerably increase the proportion of birds returning, presumably by increasing the 'area' of known country as well as the confidence of the birds. Some training was therefore needed if sufficient birds were to get back and be available for further experiments and if only competent homers were actually to be used in the tests. On the other hand, if the training was unduly prolonged there would be wastage from incidental causes. A compromise restricted training flights but increased the ground actually covered, by releasing birds up to 50 miles alternately in opposite directions. This also served to prevent the imposition of a rigid training direction, with a tendency to fly always in that direction, as has been found to be the case if such training is continued for too long. Training took place between 23 July 1953 and 23 August 1953. After releases at 1, 2 and 3 miles east, west and north, alternate releases north and south followed at each of the following distances: 6, 10, 15, 25 and 50 miles (see Fig. 4). The birds were released in groups of five up to 15 miles north, in groups of three at 15 miles south, in pairs at the 25-mile points, and singly at the 50-mile points. On days not occupied by training the birds could freely enter and leave the loft. Seventy-five birds had been reduced to fifty by the end of the training, a measure of the selection involved. These were divided into two equal lots by designating alternate ring numbers to be experimentals and controls. Consecutive numbers are given to sibs and the series run through as the squabs become ready for ringing. An equal division on the basis of heredity and age was thus ensured. Two birds returning after the division had been made were put into the control group.

In T.45 the experimentals were those birds that had been used as controls in T.44, and vice versa. For T.46, birds that had been trained from the north in 1950 were excluded. The experimental group was made up of thirteen old and two young birds that had had no previous experience of experimental conditions, and ten young used as experimentals in T.44 or T.45. The control group comprised twelve old and thirteen young birds all previously used as experimentals. The young birds in each group were equally divided according to whether it was in T.44 or T.45 that they had been used as experimentals.

The birds were given five complete days (four in T.44) of irregular light/dark alternation as indicated in Fig. 5. Their 'chronometers' having by then, it was hoped, been thoroughly upset, the birds were kept for a number of days with a regular light/dark alternation out of phase with the normal day. For the easterly

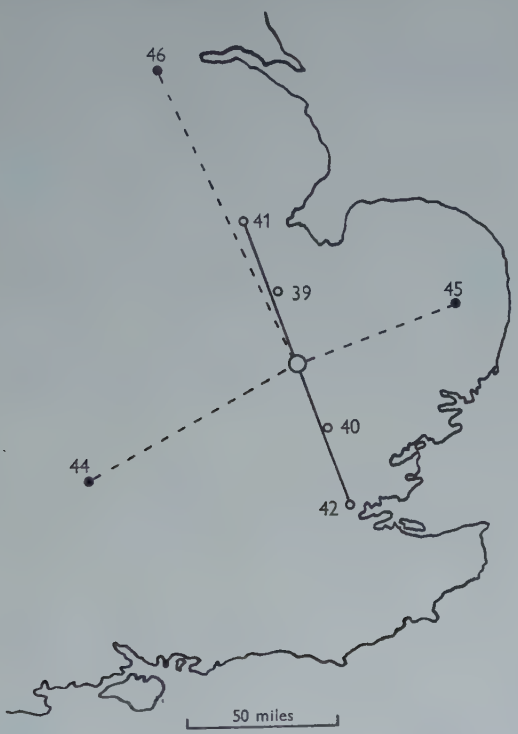

Fig. 4. Release points of young pigeons in 1953. Notation as Fig. 2.

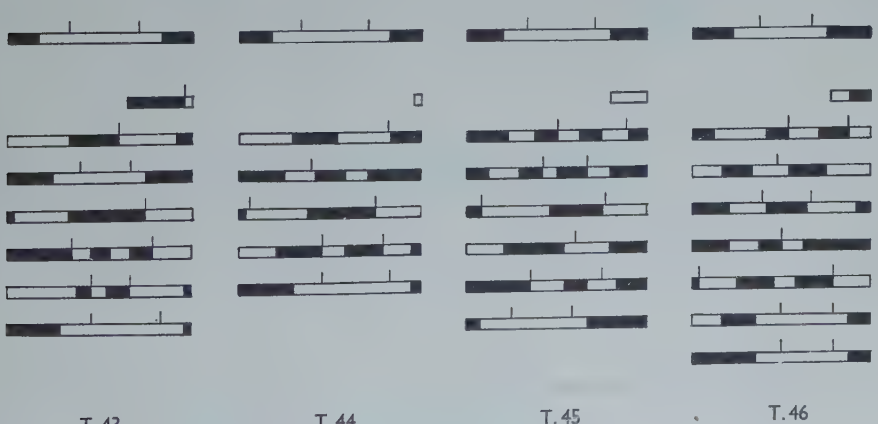

Fig. 5. Light/dark sequences (midnight to midnight) imposed in T.43–46. Upper block shows normal sequence experienced by control birds, short vertical lines indicate feeding times. Subsequent blocks show the irregular sequences and feeding times used with experimentals, with (lowest block) the first day of their regular, shifted sequence and feeding times.

releases (T.45) the artificial day was 3 hr. in advance, as in T.38. For the westerly (T.43 and T.44) and northerly (T.46) releases it was 3 hr. *behind* normal, so that, according to the theory, a *westerly* bias should be imposed. The sun at release would be in advance of the new 'chronometer' time, as would have resulted from a move far to the east. It is worth emphasizing here that to obtain statistically convincing results in field tests of this nature, it is essential to arrange for a wide divergence between true and false bearings. Even with well-orientated birds the mean deviation from the home bearing will be around 40°, with a standard deviation of about 30° (Matthews, 1953*b*). Scatters after experimental treatment which might not affect all birds in precisely the same way would be expected to be wider than this. Further, many cases are known where initial scatters have been markedly skew, even when there should be no conflict of bearings as might result from training or experiment—see, for example, Matthews (1953*a*, Figs. 2 and 4) and Kramer (1953, Fig. 5). The release points were chosen with this in mind, and were all more than 50 miles from any previous point at which the birds concerned had previously been released (Figs. 2 and 4). That for T.45 had been used in T.38 and that for T.44 in several earlier releases, T.8, 20, 35, so comparison with scatters on those occasions can be made.

The regular light/dark alternation was continued for a minimum of five days, and from then on until suitable weather conditions were forecast by the Air Ministry meteorological officers at London or Mildenhall. It is notoriously difficult to forecast if and when early fog will lift, and whether it will give clear or clouded skies. This is especially so when the information is required some twelve hours in advance so that the birds can be transported through the night (p. 42). As a result some excellent release conditions were missed, and T.43, 44 and 45*a* were all delayed in starting, because cloud or fog cleared more slowly than forecast. In T.45*a* a completely unpredicted return of heavy cloud and rain made it necessary to suspend releases when only a third complete. If the birds were kept at the release site in the van overnight, in the hope of better weather the next day, it seemed quite probable that they would pick up sufficient clues (sounds, temperature etc.) to confuse, if not to reset their 'chronometers'. It was decided to return to Cambridge, and the experimental birds were replaced in the lightproof room, under cover of darkness and, of course, in covered baskets. Since they had already been immured for 17 days, a complete repetition of the treatment could not be made. The regular lighting treatment alone was therefore resumed for another eight days. The results (T.45*b*) were so similar to the previous partial release that the two are considered together as T.45.

Results

The treatment before and conditions at release are summarized in Table 5.

The initial scatters of experimental and control birds are given in Figs. 6 and 7. A glance will show that, after the combined treatment, there is a marked difference between the two groups. This may be evaluated statistically. First, the diagrams for the controls can be bisected at right angles to the true home bearing, and the number

of birds flying towards or away from home compared with the 1:1 ratio expected on chance (Table 6). In each test the distribution is strongly non-random and orientated in the homeward direction. There is no significant heterogeneity, i.e. the results of the tests are similar to one another and can justifiably be added together in assessing the results of the whole series.

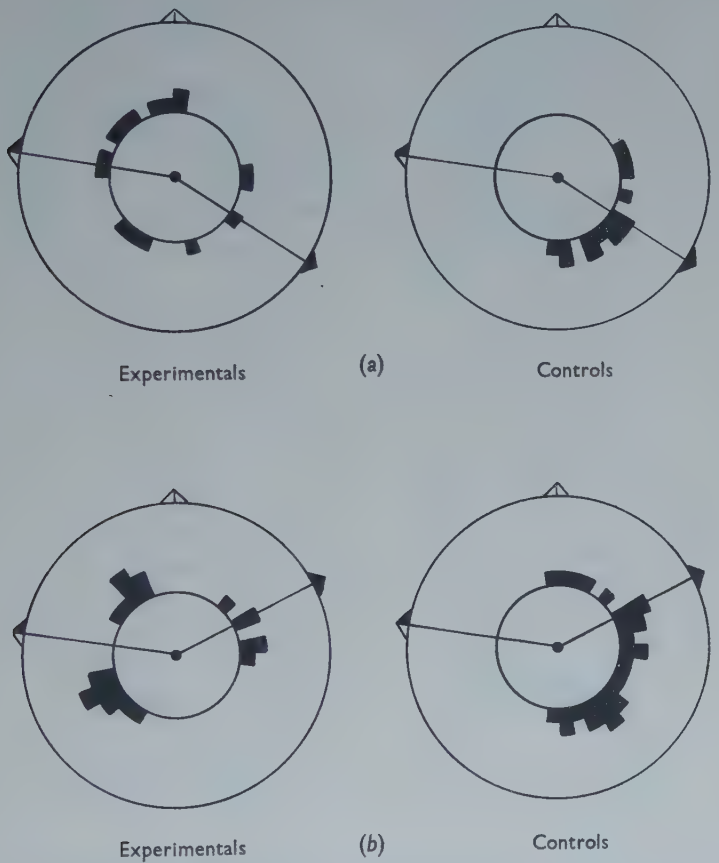

Fig. 6. Initial orientation of pigeons after treatment to disrupt and then reset 'chronometers'. (*a*) T.43, adults; (*b*) T.44, young birds.

Secondly, we can find whether the experimental distributions differ from the control distributions, by dividing both along the line bisecting the angle between home and false bearings, and comparing the numbers of birds falling into these sectors, that is, approximating more closely to one or the other bearing (Table 7). There is no question but that the experimentals were consistently giving very different scatters from those of the control birds.

Thirdly, we wish to know whether the experimental scatters differ, not only from the control, but from those which would have been expected if the birds had flown at random, as if only the first (irregular) part of the treatment had been

Table 5

Test	Days of treatment		No. of birds		Home		False bearing	Date	Time	Sun and cloud conditions	Wind
	Irregular	Regular	Exptls	Controls	Distance	Bearing					
T.43	5	5	17	17	80	123°	279°	27. viii.	12.05–15.47	Full sun, 4/10 Cu.	W. 2
T.44	4	6	25	27	80	064°	280°	6. ix.	10.45–14.58	Full sun, no cloud	E. 1
T.45*a*	5	11	8	6	56	252°	086°	27. ix.	10.39–11.51	Sun lightly veiled by high, thin stratus	N.E. 1–2
T.45*b*	5	11+8	13	16	56	252°	094°	6. x.	06.52–09.01	Full sun, no cloud	W.N.W. 2
T.46	5	10	25	25	108	158°	273°	26. x.	06.40–11.45	Full sun, no cloud	S. 2–4

Table 6

Test	$<90°$	$>90°$	χ^2	P
T.43	14	0	14·00	0·0004
T.44	21	6	8·33	0·006
T.45	20	2	14·73	0·0002
T.46	17	5	6·55	0·01
			43·61	
All	72	13	40·95	$\ll$0·0001
		Heterogeneity	2·66	0·5

Table 7

Test	Experimentals		Controls		χ^2	P
	False	Home	False	Home		
T.43	12	4	0	14	17·50	$<$0·0001
T.44	17	6	2	25	23·32	$<$0·0001
T.45	14	6	2	20	16·47	$<$0·0001
T.46	19	5	6	16	12·47	0·0007
					69·76	
All	62	21	10	75	67·92	$\ll$0·0001
				Heterogeneity	1·84	0·7

effective. To do this the experimental scatters are bisected at right angles to the false bearing, and the distribution of the birds compared with the 1:1 ratio (Table 8).

We may conclude that the experimental scatters form a homogeneous series distinctly different from random and *orientated in the false direction*, though less

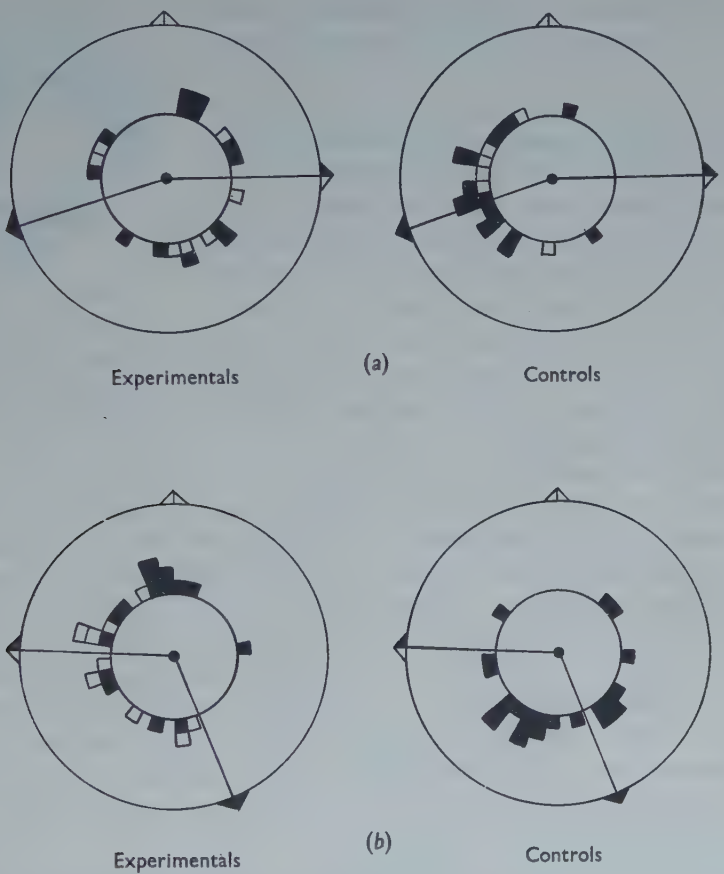

Fig. 7. Initial orientation of pigeons after treatment to disrupt and then reset 'chronometer'. (*a*) T.45, young birds; T.45*a* in white, T.45*b* in black. (*b*) T.46, adults and young birds; experimentals with previous experience of experimental treatment in white, experimentals without such experience in black.

Table 8

Test	$<90°$	$>90°$	χ^2	P
T.43	12	4	4·00	0·05
T.44	17	6	5·27	0·02
T.45	15	5	5·00	0·03
T.46	19	5	8·17	0·007
			22·43	
All	63	20	22·30	<0·0001
		Heterogeneity	0·13	>0·9

strongly than the controls were in the home direction. The combined results of all four experiments are presented in Fig. 8. The conclusion that the treatment was effective is materially strengthened by the way in which the *same* birds which orientated in the home direction when used as controls, orientated in the false direction when used as experimentals. The birds that had been uninfluenced by the treatment before T.38 were strongly influenced by that before T.43.

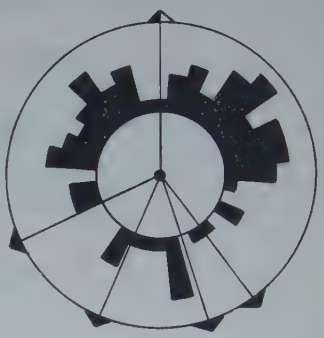

Fig. 8. Initial orientation of experimental birds in T. 43–46 as a composite diagram. All false bearings coincided and directed to top of page.

The times for which the birds were in sight showed very little variation between the groups, the average values (in minutes) being shown in Table 9. This is negative evidence that the experimentals which departed in the false direction were indeed *orientated* towards it, and not just disorientated birds which happened, by a very long chance indeed, to be lost in that sector. For it has been shown (Matthews, 1953*a*) that when pigeons are indeed disorientated, when they are released with heavy overcast, the time in sight increases significantly.

It is possible that those experimentals which approximated to the home direction did not merely represent the edge of the general scatter, but were birds whose 'chronometers' had not been altered. Their homeward tendency might then be further reinforced by their interpreting the artificial day as being due to a shift in longitude (p. 40). Inspection of Figs. 6 and 7 shows that these birds were not scattered evenly through the sector opposed to the false direction, but were clustered around the bearing of true home. In fact they give a tight 'fan' whose mean deviation, 32°, is less than that for control birds approximating to the home direction, 42°. There is also a suggestion they are above the average in orientation ability in normal circumstances. When used in other tests as controls their average deviation was still only 35°, as compared with 48° for those birds which had been falsely orientated as experimentals.

Further evidence that we are dealing with two qualitative divisions of the experimental birds comes from the study of the returns. These were rather slow for the whole series, for both experimentals and controls. This is readily understandable since (1) they had to be kept shut in for long periods, almost certainly resulting in some loss of condition, (2) the tests were continued much later in the season and moulting period than normal, (3) the starting times of three releases were delayed until relatively late in the day. This makes it more than usually difficult to place reliance on the distribution of returns as an indicator of orientation success. A dichotomy between the factors concerned in orientation and those governing the actual return has been demonstrated (Matthews, 1953*b*); for example a bird may be well-orientated and yet home slowly. There is also the risk, despite the precautions taken (p. 42), of birds from different groups joining up out of sight of the release point. Birds returning after several days have had the opportunity for

much random wandering (Wilkinson, 1952). In a narrow island like England, birds flying east or west must fairly soon reach and be diverted by a coast. If they simply continue to follow the coastline they will sooner or later reach known country—particularly, for example, in T.45. Attention should therefore be concentrated on those birds returning on the first and second days after release. The overall picture is summarized in Table 10.

Only five birds returned on the first day, four being controls and the fifth an experimental orientated homewards. Taking first and second day returns together, the table shows that the performance of those control birds which, despite their advantages, were orientated in the false direction, was the poorest of all. Then come the experimentals orientated in the false direction, followed by the homeward orientated controls. Best of all were the experimentals whose 'chronometers' were apparently unaffected and which orientated towards home. The overall

Table 9

Experimentals		Controls	
False	Home	False	Home
3·5	3·4	3·4	3·5

Table 10

Returned on days	Falsely orientated			Homeward orientated			No orientation data
	Exptls	Controls	All	Exptls	Controls	All	
1st and 2nd	23 (37 %)	0 (0 %)	23 (32 %)	13 (62 %)	38 (51 %)	51 (53 %)	4
3rd and 4th	18	4	22	4	13	17	2
Later	7	4	11	1	11	12	1
Missing	14	2	16	3	13	16	4
Total (100 %)	62	10	72	21	75	96	11

difference between birds orientated falsely and those orientated towards home is significant ($\chi^2 = 7{\cdot}49$, $P = 0{\cdot}008$), as is that between the two divisions of the experimental birds ($\chi^2 = 3{\cdot}93$, $P = 0{\cdot}05$). This confirms that we are indeed dealing with a real qualitative distinction, already suggested by the scatter diagrams.

The existence of wide variation among individual pigeons in both orientation and homing abilities has been demonstrated (Matthews, 1953*b*). It is not surprising therefore, that some birds should be less easy to confuse experimentally than others, and that these stable birds should apparently be among the best performers. The proportion of such birds amounts to 25 % in the present series, closely paralleling the results of the 1951–2 altitude change experiments (Matthews, 1953*a*) in which 18 % of the birds persisted in orientating towards home after treatment that sent the others in the opposite direction. A convincing field demonstration of the effect of experimental treatment thus depends ultimately on there being a low proportion of stable birds. Otherwise their homeward bias will prevent the scatter of the

majority in the false direction from being statistically distinguished from random. The use of abnormal reactions in an unstable part of the population in the investigation of a normal reaction is quite respectable—human psychology would be sadly handicapped without madmen and neurotics. Nevertheless this state of affairs is not wholly satisfactory, since it can lead to conflicting results between workers using different stocks of pigeons. Nor can the birds be sorted out beforehand other than by tests involving the same treatment as that employed in the main experiment. The necessity for laboratory tests in a form of solarium, in which the sun's appearance *and* movement can be accurately simulated is more than ever apparent.

The returns on the third and fourth days practically levelled out the differences between the falsely orientated and homeward orientated birds. This suggests that the effects of the treatment do not last very long, that the artificially induced alterations in the 'chronometers' may be overridden fairly easily when the birds are exposed to normal day conditions.

DISCUSSION AND CONCLUSIONS

The attempts to impose a direct shift on the 'chronometer' of shearwaters and pigeons were unsuccessful, though the possibility of an interpretation of the conditions as being due to a shift in longitude cannot be excluded. Since the present experiments were completed, Hoffmann (1953) has reported that he has altered the 'chronometers' of two starlings by keeping them for 12–18 days in an artificial day retarded by 6 hr. The new 'chronometer' setting was maintained during 28 days of constant illumination, and then returned to normal after 12–16 days in an outside aviary. The starlings were only exhibiting the simplest form of orientation, moving to food in one direction fixed by training. The change in orientation, through 90°, was that expected if the sun was simply being used as a compass. The 'chronometers' of these two birds would appear to be more easily adjustable than those of the pigeons in T.38, though a few more days' treatment of the latter might have had some effect. It now seems unlikely that successful results with the simple shift technique will be possible using wild birds taken from the nest, because of the short time available for treatment. The requirement is for a species with good homing ability, showing overt orientation at release and capable of withstanding much longer periods of captivity, either fasting or feeding readily, without diminution of the homing urge. In four days, it *may* be possible to produce a random scattering in shearwaters by irregular light/dark treatment to parallel the results with pigeons in T.35 (Matthews, 1953*a*).*

The more drastic technique aimed at disrupting and then resetting the 'chronometer' appears to have been effective. The false orientation of the majority of the birds is undeniable, and is a strong indication that longitude determination is based

* Such an experiment was made in 1954. Shearwaters were subjected to alternate light and dark periods of irregular length (28, 6, 6, 3, 7, 6, 5, 4, 8, 5, 8, 4, 15 hours) for four days. During the dark periods the record of flight calls was played. Sunny conditions obtained at release on 16 June 1954 near Cambridge, but again there was a stiff breeze (force 4) from the west. Once more controls and experimentals alike were lost downwind. There was, however, a suggestive difference in the proportion of returns—14 out of 16 controls, 8 out of 16 experimentals.

on time differences. And there does not appear to be any way in which local time could be determined except with reference to the sun arc, whose highest point is reached at local noon. This is always due south and so would provide a constant reference point when all others were lacking in completely unknown country. To this we must add the experimental evidence, also statistically satisfactory, obtained by Matthews (1953*a*) that the majority of pigeons showed a false orientation in latitude when prevented from observing the seasonal change in the sun's altitude. Against the background of a complete breakdown of orientation when the sun is hidden by overcast, these accumulated experimental data speak very strongly in favour of a form of complete sun navigation. The particular hypothesis put forward by Matthews (1951*a*) with its basic requirement of extrapolation from a short observed arc still appears to be the most plausible. Kramer's (1953) experiment would appear to have disposed of the unsatisfactory conception of sun navigation which overlooked the necessity of some reference point from which to measure the sun's co-ordinates in the instantaneous 'fix' it proposed.

SUMMARY

1. An investigation was made of that part of the sun navigation hypothesis which proposes that birds detect longitude displacement by comparing home time (provided by an internal 'chronometer') with local time (estimated from the highest point of the sun arc).

2. Shearwaters were exposed for 4 days, and pigeons for ten days, to an artificial day 3 hr. in advance of normal. This did not result in any confusion of their orientation when released to the east.

3. More drastic treatment was then used, pigeons being subjected to 4–5 days of irregular light/dark sequences, followed by 5–11 days of regular sequences, advanced or retarded with respect to normal.

4. In tests from the west (2), east and north after this treatment, the 'chronometers' had apparantly been affected and the birds showed a definite tendency to fly in the predicted false direction—east after an advanced day, west after a retarded one.

5. Variations in the time-in-sight, and in the proportion of the more rapid returns supported the conclusions drawn from the orientation data. In a minority (25%) of the birds, the evidence suggests that the 'chronometers' were not affected.

6. It is concluded that these new results, taken with those produced previously, strongly support the suggestion that a form of complete, bico-ordinate sun navigation is used by birds.

It is a pleasure to again acknowledge my indebtedness to Prof. Sir James Gray for making the continuation of this work possible, and to Dr W. H. Thorpe for his constant interest and encouragement. The pigeons were maintained most efficiently by G. E. Dunnett, assisted by Miss E. M. Barraud. The shearwater experiments

were made possible by the co-operation of the Council for the Promotion of Field Studies, their Wardens, J. H. Barrett and P. J. Conder, and their staffs and visitors.

The recording of shearwater flight calls, made by Dr Ludwig Koch, was donated by the B.B.C.

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STUDIES ON THE MYONEURAL PHYSIOLOGY OF ECHINODERMATA

II. CIRCUMORAL CONDUCTION IN *CUCUMARIA*

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The properties of a neuromuscular preparation of the mid-ventral pharyngeal retractor muscle of *Cucumaria sykion* (Lampert) have been previously described (Pople & Ewer, 1954). When excited by stimulation of the radial nerve, this muscle shows a double response consisting of a quick component, tentatively attributed to direct nervous paths through the motor complex of the muscle and a delayed component which possibly arises from secondary paths within the motor complex itself. This characteristic double response can be clearly seen in the contractions recorded in the upper trace of Fig. 2 of the present paper. The tension developed by this muscle is dependent upon the intensity of stimulation applied to the radial nerve. No evidence of frequency sensitive facilitation at the myo-neural junction was found.

The present paper is concerned with a study of the responses of the retractor muscles to impulses conducted around the circumoral nerve ring. For this purpose two types of preparation have been used.

Double-muscle preparation. This consists of the mid-ventral and left ventral retractor muscles and their corresponding radial nerves together with the section of the circumoral nerve between the two muscles. The method of preparation is similar to that previously described for the single muscle preparation. To prevent rupture of the circumoral nerve it has been found best to hold the preparation down with a piece of plastic sheeting stretched over the ossicles and the sector of the pharyngeal mass.

Five-muscle preparation. This consists of all five retractor muscles. Five cuts are made from the cloaca anteriorly through the inter-radial integument to the lip of the oral cavity. The viscera and the longitudinal strips of integument posterior to the retractor muscles are cut away, leaving the pharyngeal mass and its five retractor muscles. The five radial nerves which lie beneath the longitudinal radial muscles are dissected out as in other preparations. The preparation is impaled upon a glass spike passing through the oral lip, oral cavity and oesophagus and is prevented from twisting by a star-shaped piece of plastic sheeting, the points of which pass between the muscles and are held down by pins.

Light auxotonic levers were used throughout. The tension developed by a muscle depends upon the initial stretch. In most preparations the levers were adjusted so that all muscles were stretched to the same initial length; this was not however always done (e.g. Fig. 4). The lever system was usually adjusted so that a shortening

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of 1 mm. developed a tension of 1 g. Fine platinum wire electrodes were used. The cathode was placed beneath the radial nerve and the anode upon the longitudinal radial muscle immediately above the cathode. The electrodes were normally placed 0·2–0·5 cm. from the motor complex. All experiments were carried out at temperatures of 20–23° C.

THE CHARACTERISTICS OF CIRCUMORAL CONDUCTION

The general anatomical organization of a double muscle preparation is shown in Fig. 1. If a suitable stimulus is applied to the radial nerve *a* at *E* both muscles *A* and *B* will contract. Nerve impulses to the ipse-radial muscle *A* will pass along the radial nerve, the motor nerve and through the motor complex α. Impulses to the para-radial muscle *B* will pass along the radial nerve to its point of union with the circumoral nerve *R*, along this to the next radial nerve and then along the radial nerve *b* to the motor nerve and motor complex β. A comparison of the responses of muscles *A* and *B* will therefore give information about the characteristics of circumoral conduction. Histologically the circumoral and radial nerves are composed of the same neural elements, as Smith (1937) has shown in *Marthasterias glacialis* (L.).

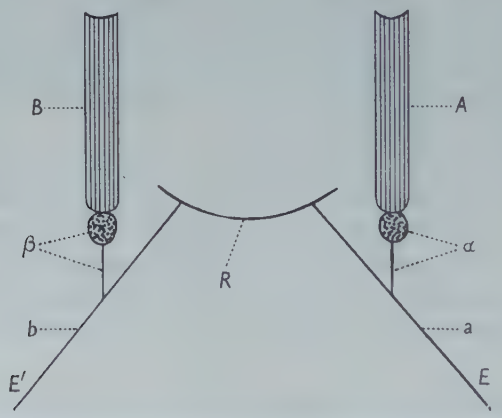


Fig. 1. Diagrammatic representation of the innervation of two adjoining retractor muscles *A* and *B*. *a*, *b*, radial nerves; α, β, motor nerves and motor complexes; *E*, *E′*, points of stimulation; *R*, circumoral nerve.

Effect of intensity. If, with such a preparation, a single shock well above threshold is applied to the radial nerve *a*, both ipse-radial and para-radial muscles will contract. Both responses show the quick and delayed components previously described in the account of a single muscle preparation. If the stimulus intensity is lowered until it is just above threshold the ipse-radial muscle will respond with a small contraction, while the para-radial muscle may show a minute response. With increasing intensities of stimulation the tensions developed by both muscles increase until a maximum is reached. The same result is obtained with batteries of stimuli (Fig. 2). It will be noted that the maximal tension developed by the para-radial muscle is never as great as that which this muscle can develop with supra-maximal stimulation

of the radial nerve of its own radius. Further, it may be seen (Fig. 3) that although the tension developed by the para-radial muscle is less than that developed by the ipse-radial muscle, both achieve maximal tension at about the same intensity of stimulation of the radial nerve.

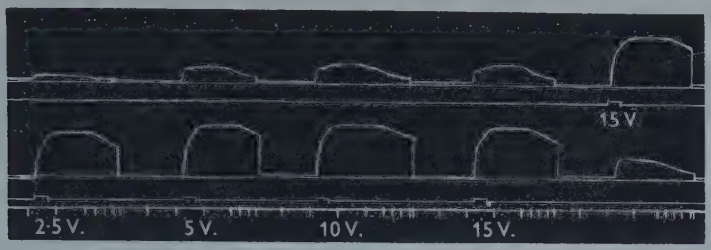


Fig. 2. *Cucumaria* pharyngeal retractor muscles. Responses of two adjoining pharyngeal retractor muscles recorded one above the other. Volleys of stimuli of various intensities are applied to one radial nerve. In the first four responses the radial nerve supplying the muscle recording the lower trace was stimulated; in the last response the stimulus was applied to the radial nerve supplying the muscle recording on the upper trace. Stimulation frequency 2·5 s./sec. Ten stimuli in each battery of shocks. Time marker 10 sec.

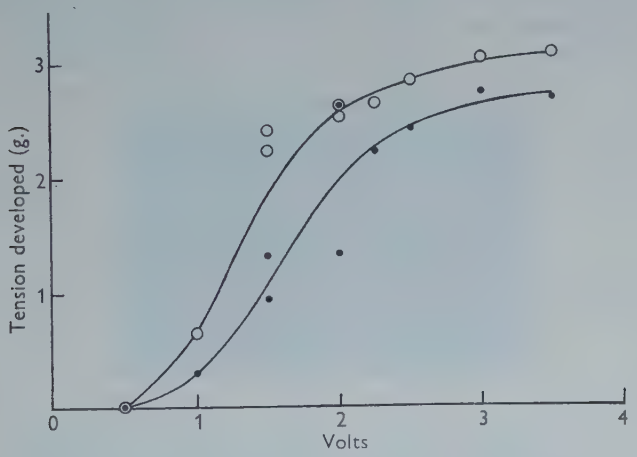


Fig. 3. Graph showing the relation between stimulus intensity and tension developed in an ipse-radial muscle (open circles) and the adjoining para-radial muscle (full circles). When stimulated by way of its proper radial nerve the para-radial muscle developed a maximal tension of 4·75 g. Stimulation with 10 shocks of 2 msec. duration at a frequency of 2·5 s./sec.

Effect of frequency. If the frequency instead of the intensity of stimulation is altered, it is found that the responses of the para-radial muscle change in a similar manner to those of the ipse-radial muscle. This effect is shown in Fig. 4. At very low frequencies of stimulation both muscles respond with incompletely fused contractions; at 1 stimulus/sec (s./sec.) the response is almost smooth and smooth contractions, neglecting the humping caused by the double character of the response, are obtained at 2·5, 5 and 10 s./sec. At 50 s./sec. the tension developed by both muscles is less than at 5 s./sec. This depression of the responses at high frequencies of stimulation has already been described in the single-muscle preparation.

Conduction velocity. It is possible to make an approximate assessment of the conduction velocity of the impulses releasing the quick response in their passage along the circumoral nerve. It is, however, necessary first to show that the inter-radial paths do not pass by way of the motor complex. Thus in Fig. 1 impulses originating at *E* might pass directly along the radial nerve to the circumoral nerve or relay

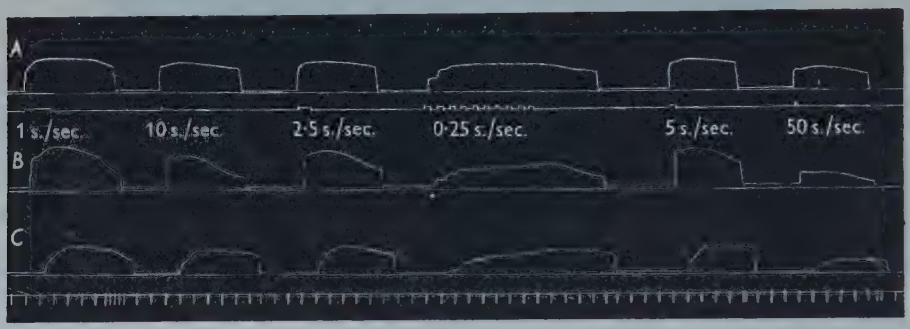


Fig. 4. Extract from a five-muscle preparation. The radial nerve of the mid-ventral muscle, recorded on the upper trace, was stimulated with batteries of 10 stimuli of 2 msec. duration at different frequencies. The two lower traces record the responses of the right ventral (*B*) and right dorsal (*C*) muscles. The muscles have been unequally stretched so that the lever excursions recorded by each are approximately equal. Stimulus intensity 8 V. Time marker 5 sec.

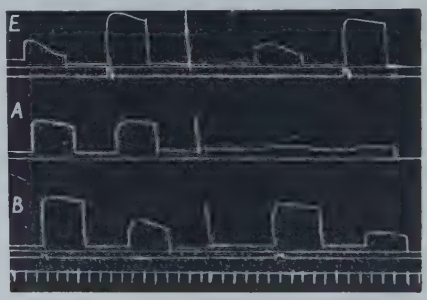


Fig. 5. Records from the mid-ventral (*A*), left ventral (*E*) and right ventral (*B*) retractor muscles. In the first two responses the radial nerve to *B* and then to *E* is stimulated as shown by the relevant signal markers. The motor nerves and motor complex of *A* were then almost completely destroyed by cautery and the radial nerves of *B* and *E* again stimulated. Stimulus in each case a battery of 10 shocks of 2 msec. duration at 15 V. Time marker 10 sec.

through the motor complex α. Similarly, impulses passing from one radius to a more distant one might relay through each succeeding motor complex in turn. That this does not occur is demonstrated in Fig. 5. In a preparation of the mid-ventral, right and left ventral muscle two test shocks were applied; first to the left and then to the right ventral radial nerve. The motor nerve and complex of the mid-ventral muscle were then destroyed almost completely by cautery. It can be seen that the cauterization does not markedly affect the transmission between the right and left ventral radii. Similarly, it may be shown, in a double-muscle preparation, that destruction by cautery of one motor complex does not affect the responses from the para-radial muscle to stimulation of the radial nerve whose motor complex has been

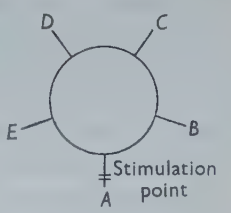
destroyed. It may be taken then that the path of excitation from one radius to another more distant passes only by way of the radial and circumoral nerves without relay through the motor complex.

The velocity of conduction along the circumoral nerve may be determined in a three-muscle preparation. Calling the three radii *A*, *B* and *C*, a stimulus is applied to radial nerve *A* and the difference in latent period of response between muscles *B* and *C* is determined. This time difference will be the time taken for conduction around one-fifth of the circumoral nerve ring. Measurements made on seven preparations gave a mean value of $0{\cdot}11 \pm 0{\cdot}04$ m./sec. at 20–23° C. It must be emphasized that this value is very approximate as considerable uncertainty attaches to determinations of the length of the nervous tracts owing to the contractility of the tissues around them. This value may be compared with that of $0{\cdot}17 \pm 0{\cdot}03$ m./sec. previously determined for the conduction velocity along the radial nerve of impulses releasing the quick response. It is clear that the velocities are of the same order.

As has been previously emphasized, determination by mechanical methods of the conduction velocity of the impulses releasing the delayed response is difficult. Two determinations made previously suggested that there was no significant difference between the conduction velocities along the radial nerve of the impulses releasing the two types of response. Evidence that these conduction velocities are also of the same order along the circumoral nerve ring is obtainable with a five-muscle preparation. If a strong stimulus or a battery of stimuli be applied to one radial nerve, all five muscles will contract. The time between the onset of the quick and delayed responses is found to be almost the same in all five muscles (Table 1). Certainly there is no evidence that the interval is greatest in the muscles most distant from the stimulated radius.

Table 1. *Time delay between the onset of the quick and delayed responses of the muscles of a five-muscle preparation when a supra-maximal stimulus is applied to the radial nerve of one muscle*

Muscle	*A*	*B*	*C*	*D*	*E*
Delay (sec.)	$2{\cdot}1 \pm 0{\cdot}3$	$2{\cdot}9 \pm 0{\cdot}3$	$2{\cdot}1 \pm 0{\cdot}3$	$2{\cdot}0 \pm 0{\cdot}6$	$2{\cdot}6 \pm 0{\cdot}3$



Delayed facilitation. In the single-muscle preparation a delayed facilitation of the slow response was described. If two single stimuli are applied at intervals up to about 60 sec. the magnitude of the delayed response to the second stimulus is greater than that to the first.* Evidence has been presented which suggests that the site of this facilitatory effect lies in the motor complex. The phenomenon may be analogized with post-tetanic potentiation. A similar facilitation of the delayed

* This effect is not found for all preparations and may appear in only one or two muscles of a five-muscle preparation. The cause of this variability is being investigated.

response of the para-radial muscle in a double-muscle preparation may also be demonstrated. Moreover, it is possible to facilitate the response given by a muscle to stimulation of its own radial nerve by previous stimulation of the para-radial nerve and vice versa.

From these experiments it appears that conduction around the circumoral ring is very similar to that along a radial nerve. In both cases a muscle shows quick and delayed responses; in both tension recruitment is dependent upon the intensity and not the frequency of stimulation; in both delayed facilitation is found and the conduction velocities of impulses are of the same order. These findings are not unexpected as the radial and circumoral nerves are histologically similar. One important point, however, does emerge, namely, that in these conditions stimulation never releases maximal possible tension from a para-radial muscle. In other words conduction around the circumoral ring appears to be 'decremental'.

DECREMENTAL CONDUCTION

If in a five-muscle preparation the mid-ventral radial nerve is stimulated at an intensity above threshold all five muscles contract. The tension developed by the right and left ventral muscles is less than that developed by the mid-ventral muscle, while that developed by the right and left dorsal muscles is smaller still. That this effect is symmetrical around the ring can be seen in Fig. 6*a* where a supra-maximal shock was applied to each radius in turn.

Fig. 6*b* shows responses from the same preparation after cutting the circumoral ring between radii *A* and *E*. A complete series of decremental stages around the ring may be seen in the responses obtained on stimulating radial nerves *A* and *E*. In trace 6*b* a minute response is given by muscle *A* when the radial nerve supplying muscle *E* is stimulated. No corresponding response from muscle *E* after stimulation of radial nerve *A* was recorded in this case, but the examination of results from further preparations shows that complete transmission does frequently occur. Moreover, it will be seen that the response obtained from stimulating nerve *C*, which lies immediately opposite the cut, is almost unchanged.

As with the double-muscle preparation, all five muscles respond when a single shock is applied to one radial nerve and the tensions developed in each of the five muscles depend upon the intensity and not the frequency of stimulation applied to any one radial nerve.

The simplest interpretation of these various results which can be offered is that the decremental effect depends simply upon the geometrical arrangement of the fibre tracts running from a radial nerve to its own and other motor complexes. This is illustrated in diagrammatic form in Fig. 7. From radius *A* will run, say, 10 motor tracts to the motor complex α; to β will run, say, 6 directly anti-clockwise and one clockwise; to γ 3 anti-clockwise and 2 clockwise and so on. The maximal responses from the five muscles to stimulation of radial nerve *A* should then be in the following proportions: *A*, 10; *B* and *E*, 7; *C* and *D*, 5. If now a cut is made between *A* and *E* the expected responses will be *A*, 10; *B*, 6; *C*, 3; *D*, 2; and *E*, 1. Examination of Fig. 6*a* and *b* shows that this expectation is broadly confirmed. Thus when radial

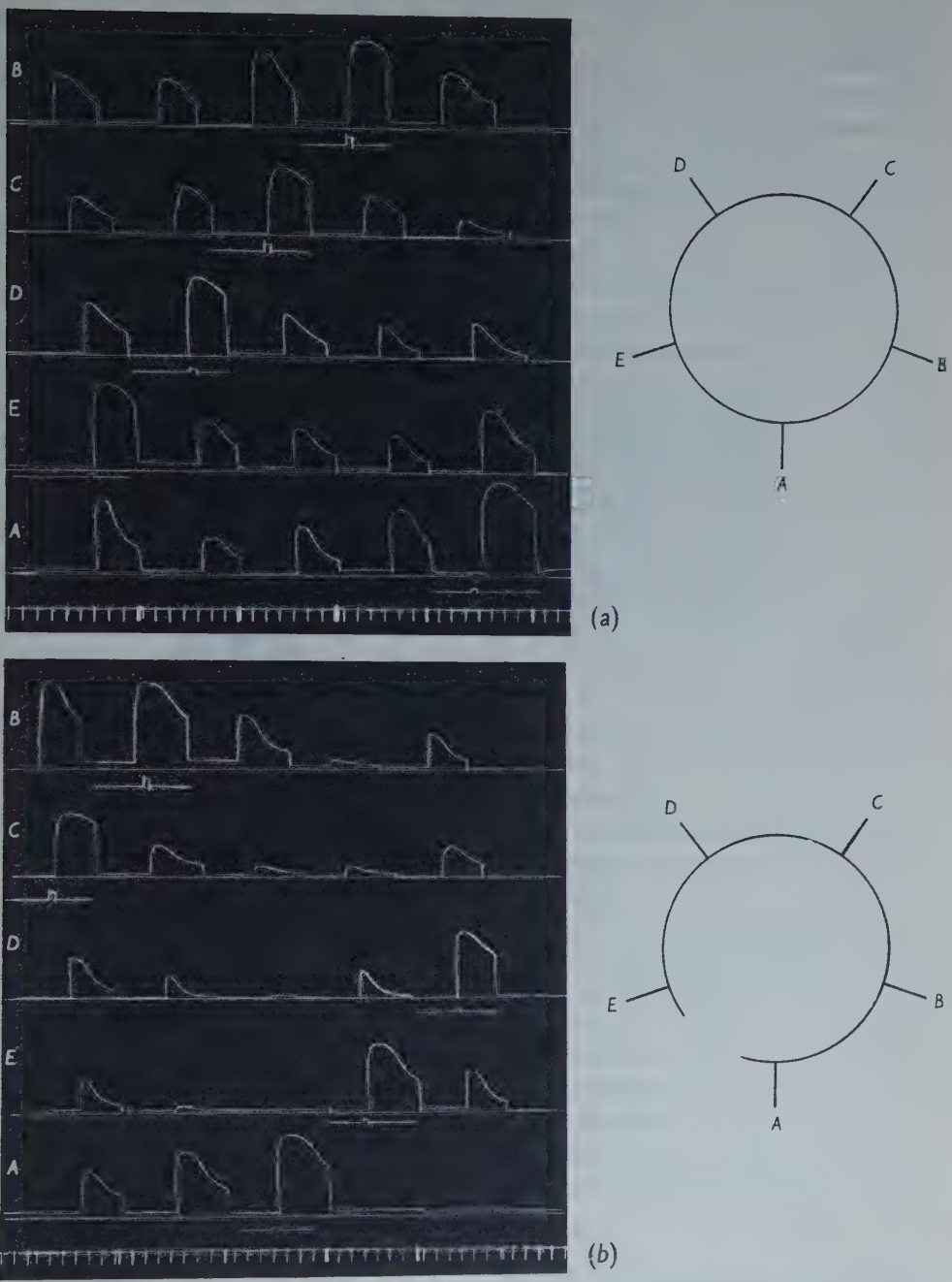


Fig. 6. Records from a five-muscle preparation in which a supra-maximal stimulus is applied to each muscle in turn. Records below the traces indicate which radial nerve is stimulated. *a* with the radial nerve intact; *b* after the radial nerve has been severed between the muscles *E* and *A*. In all cases 10 stimuli at 2·5 s./sec. Intensity 10 V., duration 2 msec. Time marker 10 sec.

nerve *A* is stimulated, muscles *C* and *D* give weaker responses after section of the nerve ring. Similarly, when radial nerve *E* is stimulated the same effect can be seen with muscles *C* and *B*.

If such a picture is correct it will be expected that when the ring is intact evidence of the separate arrival of impulses which have passed clockwise and anti-clockwise around the ring should appear. This is occasionally observed in muscles which develop tension rapidly. The normal slow development of tension usually obscures the effect.

In the previous paragraphs it has been assumed that there is no convergence of the two sets of fibre tracts which supply the ultimate axons to produce the quick response or of those supplying the neurones within the motor complex to produce the delayed response. Thus, for example, it is assumed that the response from muscle *C* (Fig. 7) supplied by the motor complex γ will be proportional to the number of fibres entering the motor complex by clockwise and anti-clockwise paths. This assumption is not fully justified.

Fig. 7. Diagrammatic representation of the suggested arrangement of circumoral tracts arising from a single radius. The thickness of the lines is proportional to the number of nerve fibres. α, β, γ, etc., represent the motor complexes of the five pharyngeal retractor muscles. For further explanation see text.

If, in a double-muscle preparation, as illustrated in Fig. 1, a supra-maximal stimulus is applied to radial nerve *E* and simultaneously a supra-maximal stimulus is applied to *E'*, the response from muscle *A* is no greater than when a single supra-maximal shock is applied to *E*. This effect is shown in Fig. 8. This suggests that the ipse-radial and para-radial fibre tracts do converge. With submaximal stimuli some summation occurs but the response to simultaneous stimulation is never greater, and usually is rather less than the algebraic sum of the separate responses to ipse-radial and para-radial nerve stimulation. These effects are found with both quick and delayed responses. The presence of such convergence necessitates some modification in detail to the system suggested tentatively in Fig. 7. It does not, however, offer an alternative explanation of the decremental effect since the latter persists after the nerve ring has been cut. Each motor complex then receives impulses from one set of fibres only and no convergence enters into the system.

An alternative explanation of the absence of complete algebraic summation when both nerves are stimulated simultaneously could lie in the presence of inhibitory nerves running from radial nerve *b* (Fig. 1) to the para-radial motor complex α and here partly inhibiting the effects of motor impulses arising from the radial nerve *a*.

This more complicated explanation cannot be excluded. It is found that if a stimulus is applied to E' and then subsequently to E the summed response from muscle A is no greater than when both nerves are stimulated simultaneously. Even when the interval between the two stimuli is as great as 10 sec. no difference in the magnitude of the summed responses is found. It seems then that if there are inhibitory nerves running between radii, their effect must persist for remarkably long times.

The 'decremental' effects shown in Figs. 2 and 6 might also be interpreted in terms of inhibitory nerves. The simplest relation would then be that from one radial nerve there ran to each motor complex in both clockwise and anti-clockwise directions equal numbers of motor nerves and varying numbers of inhibitory nerves. The inhibitory innervation pattern would then be the reverse of that suggested in Fig. 7. There is no evidence in favour of such a complex hypothesis, but

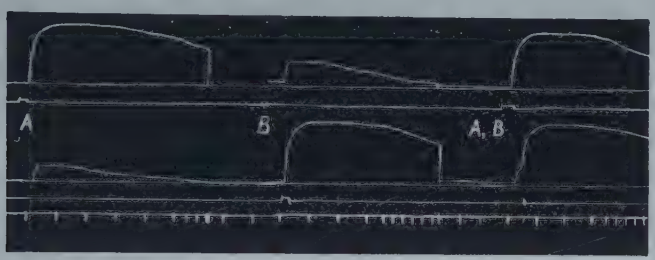


Fig. 8. Records from a double muscle preparation. A supra-maximal stimulus is applied first to one radial nerve, *A*, and then to the other *B*. Both radial nerves are then stimulated simultaneously, *AB*. Note that when both nerves are simultaneously stimulated the responses from the muscles are no greater than when only the radial nerve proper to the muscle is stimulated. In all cases 5 stimuli at a frequency of 2·5 s./sec. and intensity of 5 V. were applied. Time marker 10 sec.

if it be indeed the physiological basis of the observed decrement, its characteristic effects depend, as in the simpler explanation, upon the geometrical organization of fibre tracts and not upon some special physiological peculiarity of the synaptic structures.

One final point warrants comment. From a consideration of the dominance of different radii in the walking of a starfish, Smith (1945, 1950) suggests that the anatomical basis of the inter-radial circumoral tracts is such that from a single radius two tracts flow clockwise to the two left para-radii and two anti-clockwise to the two right para-radii. The circumoral connexions from any radius are organized like a horseshoe. However, the organization of the inter-radial tracts of *Cucumaria* which supply the retractor muscles is different since they run both clockwise and anti-clockwise to the most distal radius. There is of course no reason to assume that the two types of postulated organization are in any way mutually exclusive.

DISCUSSION

In general these experiments show that the characteristics of conduction around the circumoral nerve tracts supplying the pharyngeal retractor muscles of *Cucumaria* are very similar to those found in the radial nerve. The decremental conduction is, however, a distinct property for which no clear evidence was found in the radial

nerve. It is a known characteristic of echinoderm preparations (Smith, 1945) and has previously been explained in different ways.

Smith (1945, 1950) has suggested that the properties of the echinoderm epidermal network are similar to those of the nerve nets of sea anemones studied by Pantin (1935). Apparent decrement in anemones depends upon synaptic facilitation and is influenced by both the number and frequency of stimuli applied. We have found no evidence for such an effect in *Cucumaria*. All muscles of a five-muscle preparation respond to a single stimulus applied to a radial nerve and the characteristics of this response are not modified by repetitive stimulation in any manner suggesting the presence of frequency-facilitated synapses. These results with *Cucumaria* do not however exclude the possibility of such an effect in epidermal nerve nets.

Kinosita (1941) has studied the behaviour of sea-urchin spines and offers a different interpretation of decremental phenomena. He suggests that nerve tracts run out radially from the muscles around the base of each spine to the muscles of adjoining spines. There are usually no synaptic junctions between these different fibres. Propagated responses over the test depend upon proprioceptive relay mechanisms. If the muscles on one side of a spine are stimulated to contract, this movement stretches the muscles on the opposite surface of the spine. This stretch in turn stimulates nerve fibres running to the muscles at the bases of adjoining spines and the response thus spreads over the test. These proprioceptive links do not respond in an all-or-nothing manner and this is regarded by Kinosita as the origin of diffuse and decremental responses.

Translated into terms of the retractor muscle system of *Cucumaria* this would imply that relay around the circumoral ring would depend upon proprioceptive links between succeeding retractor muscles. No evidence of such links has been found. In a five-muscle preparation neither the stretching of a single muscle nor direct electrical stimulation applied to a single muscle causing it to contract produces any response from the other four muscles. Furthermore, as shown in Fig. 5, excitation will pass unchanged beyond a radius in which the response of the muscle has been almost completely eliminated by destruction of its motor nerve and motor complex. Clearly the system envisaged by Kinosita does not account for the decremental conduction here observed. This, of course, does not exclude the possibility of a different mechanism occurring in the epidermal muscular systems of echinoids.

Our results suggest that the decremental effects in circumoral conduction which we have observed are due simply to the spatial organization of nerve fibre tracts. The general similarity between the properties of the circumoral and radial nerves supports Smith's (1950) suggestion that the circumoral tracts serve mainly to connect the radii and correspond in no way to a 'central nervous system'. The anatomical basis of 'central nervous' functions in an echinoderm probably lies in its numerous and scattered motor complexes.

SUMMARY

1. The general characteristics of circumoral nervous conduction in *Cucumaria* have been studied by the use of preparations consisting of the retractor muscles and radial nerves of two adjoining radii joined by a sector of circumoral nerve ring and

by the use of similar preparations of all five retractor muscles and the complete circumoral nerve ring.

2. The characteristics of the responses of muscles stimulated by way of circumoral nerve tracts are as follows: the muscles respond with a quick and a delayed response; the magnitude of these responses depends upon the intensity of stimulation applied to an adjoining radial nerve, but is unaffected by frequency of stimulation up to a rate of 10 s./sec.; at high frequencies of stimulation both quick and delayed responses are depressed; the conduction velocity of impulses releasing quick and delayed responses is of the same order; the delayed response may show a prolonged facilitation previously analogized with post-tetanic potentiation. In these characteristics the muscular responses to impulses conducted in the circumoral nerve tracts are similar to those found to impulses conducted in the radial nerve tracts alone.

3. When, in a preparation of the five-retractor muscles, a radial nerve is stimulated, the muscles of radii nearer the stimulated nerve contract more strongly than those of radii further away.

4. Evidence is presented in favour of the view that this 'decremental' effect is dependent upon the geometrical arrangement of the fibre tracts in the circumoral nerve. The effect is not dependent upon frequency-sensitive synaptic junctions nor upon proprioceptive relays.

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Note added in proof. Shortly after this paper went to press an important study by G. A. Kerkut (The mechanisms of co-ordination of the starfish tube feet. *Behaviour* (1954), **6**, 206–32) appeared. In this evidence is presented that, contrary to Smith's (1945, 1950) interpretation, in the starfish excitation can pass from one radius around the circumoral nerve ring to the most distal radius. This agrees with our findings in *Cucumaria*.

AN INVESTIGATION OF HOMING ABILITY IN PIGEONS WITHOUT PREVIOUS HOMING EXPERIENCE

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INTRODUCTION

Recent experimental studies of the homing behaviour of birds, particularly of the pigeon, have sharply focused scientific attention upon this age-old problem. Evidence that pigeons released in strange territory beyond visual range of familiar landmarks are able to turn promptly toward home, regardless of the direction, was first reported by Matthews (1951). He used birds that had been trained by repeated releases in one direction from the loft, and his critical homing releases were made by taking the birds approximately 80 miles in a different direction. Kramer & St Paul (1951), working before Matthews published his report and without any knowledge of his results, also found homeward orientation upon release. However, they did not use directional training, but released pigeons at 200 miles, the birds having previously made only a few 10-mile homing flights from different directions. In both investigations the pigeons, as observed from the release point, showed a tendency to start toward home, and most of them homed successfully, some within a remarkably short time (e.g. 200 miles in $7\frac{1}{2}$ hr.).

The primary objective of the investigations covered by this report was to test the homing ability of pigeons on their first long-distance release (75–147 miles) when this was the first time they had been set free out of sight of the loft. In a recent investigation of homing ability in gulls, Matthews (1952) observed that a migratory species, the lesser black-backed gull, showed a statistically significant tendency to leave the release point in the home direction. The herring gull, a non-migratory species, on the other hand, showed no such tendency. This fact suggests that a non-migratory bird may be poor at orienting toward home upon its first release. The question raised was whether completely inexperienced pigeons, which range over a much smaller home territory than does the herring gull, would likewise show random departures, or whether they would demonstrate a homing ability that is completely unlearned.

After the present series of experiments was completed, I learned that Matthews had been investigating the same problem (1953*b*). Matthews made four sets of releases of untrained pigeons. The first one, involving five birds that had not been made accustomed to the crates, gave no evidence of homing orientation. The other three tests were made with birds that had previously spent several nights in the crates and after each such confinement were released the next day within 150 yards

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of the loft. These birds did reveal a statistically significant tendency to leave the release point (50 or 75 miles) in the general direction of the loft. The success in returning to the loft, however, was far below the level Matthews found in his releases with trained birds. Only one untrained bird out of a total of thirty-nine released reached home.

The results from untrained birds to be presented in this report confirms Matthews's findings regarding the orientation toward home at the release point. They differ from his on the point of homing success, as a much higher percentage of my untrained birds were successful in regaining the loft.

PROCEDURE

This report includes the results of experimental releases made on three occasions. Each set of releases involved different birds, approximately half of them having had no homing experience before they were used in the experiment (untrained group), and the others, birds with homing experience limited to three short-distance releases during the 2 weeks preceding the test release (trained group). The first experiment consisted of seven releases at the Bishopville, South Carolina, fire lookout tower on 5 March 1953 ($\text{Bishopville}_{\text{II}}$), with one, two, or three birds in each release.* The bearing of the loft was 31°, and the distance, 143 miles. The other two experiments were from the Hoffman, North Carolina, fire tower, a distance of 75 miles from the loft and with a home bearing of 28°. $\text{Hoffman}_{\text{I}}$ took place on 28 March 1953, and $\text{Hoffman}_{\text{II}}$ on 1 August. Single-bird releases were used in the two Hoffman experiments; fifteen birds in $\text{Hoffman}_{\text{I}}$ and twenty-nine birds in $\text{Hoffman}_{\text{II}}$.

When the birds were selected for the three experiments, certain ones were designated to receive three short-distance homing flights, and the others to receive only treatment intended to accustom them to being handled, followed by releases at the loft. For convenience, these will be referred to as the 'trained' and 'untrained' groups, respectively.

Before each of the three periods of treatment, the birds were picked up in the loft during the preceding night. Both groups of birds received the same amount of handling and approximately the same period in the crates on each occasion. When the trained birds were placed in the trunk of the car to be hauled away for a preliminary homing flight, the crates containing the untrained birds were placed on the ground in view of the loft (not more than 10 yards away) where they remained until the first of the trained birds reached home. The untrained birds were then simply released at the loft, usually in a group. The distance of the short homing flights before $\text{Bishopville}_{\text{II}}$ was 10 miles, and the home directions were north-east, north-west, and south, successively. In the two Hoffman experiments, the distance was reduced to 2–3 miles, with east, west, and south being the directions toward the loft.

* Each test is designated by the name of the tower used as an observation point, with a Roman numeral subscript to indicate the particular occasion of using the tower concerned. $\text{Bishopville}_{\text{I}}$, a pilot experiment conducted on 31 January 1953, while Dr Gustav Kramer was visiting Duke University for the purpose of teaching me his method of working with pigeons, has been summarized elsewhere (Pratt, 1953).

The birds were hauled to the release point under conditions that eliminated any clues to the position of the sun, which might have provided an indication of the direction of displacement. In Bishopville$_{II}$ the crates were in the back seat of a car covered with a double thickness of canvas. In Hoffman$_{I}$, they were in the trunk of the car covered with canvas and with the lid raised only half an inch. In Bishopville$_{II}$, when more crates were necessary, they were also hauled in the trunk, wrapped about with both cardboard and canvas and with the trunk lid lowered as far as the crates would allow.

The general plan at the release point was to let the untrained birds go before the others. This procedure served two purposes. (1) Any homeward orientation shown by the untrained birds in their departure flights could not be attributed to their having observed the birds with previous homing experience. (2) It increased the chances that the untrained birds would reach home ahead of the others provided they had the ability and motivation to complete the journey, and thus offered a hope that the results might indicate whether training is essential for success in regaining the loft.

This general rule of letting all the untrained birds loose first was modified in Hoffman$_{I}$ to a slight extent. In that experiment birds from two different lofts were used, one loft group consisting of six birds of 4 months of age (Duke stock), and the other, nine birds of approximately 7–9 months of age (Cranford stock). The two lofts thus provided an opportunity to compare birds differing both in heredity and in age. There were trained and untrained birds from each loft, and all of the younger birds were released before any of the older ones were set free. There were three releases of untrained birds followed by three of trained birds from the Duke loft, and then there were three releases of untrained birds followed by six of trained birds from the Cranford loft. The two lofts were 2 miles apart, and they were in the same direction from the release point.

Hoffman$_{II}$ also involved a comparison of birds of different ages and hereditary strains, but all of them had been settled in one loft when they were about 28 days of age. In this experiment fourteen trained and fifteen untrained birds were used. After the fifteen untrained birds were released, a delay of an hour was made before starting the releases of trained birds. The object was to increase the likelihood that some of the untrained birds might complete the homeward journey before the first trained bird.

The release cage was set up facing in different directions for successive releases. It had a sliding panel for one wall which was lifted by hand to set the bird free. Each bird was watched through field glasses by at least two observers, and the interval from the moment of release until the bird vanished from sight was recorded. The vanishing point was marked visually by some landmark before the glasses were moved, and the azimuth degree reading of the landmark was found as showing where the bird disappeared from view. Each observer also charted from memory the path the bird followed.

The birds awaiting release were all kept under cover. The bird for the next flight was not placed in the release cage until at least 5 min. after the previous bird had gone out of sight.

Observers at the loft recorded the time of arrival of the birds homing on the day of release, and the loft was checked for later arrivals on the following days.

Other experimental conditions were introduced to test their possible effects upon homing orientation. These will be described later in connexion with the actual findings to which they are related.

WEATHER CONDITIONS

On each release day, uniform weather conditions existed over the area including the release point and the home loft. All three tests were made with clear skies or with thin clouds permitting a clear view of the sun. The following records were made at the release points.

Bishopville$_{II}$: scattered cirrus clouds. Wind from west at 15 m.p.h., occasional gusts to 25 m.p.h. Temperature from 40–55° F.

Hoffman$_{I}$: cirrus clouds covering approximately three-tenths of sky. Wind from west, 6 m.p.h. Temperature 55–70° F.

Hoffman$_{II}$: clear, winds variable, east to north, at 4–12 m.p.h. Temperature 80–94° F.

RESULTS

Orientation at the release point

The vanishing points of the birds for all three experiments are shown in Fig. 1. Each radiating unit block represents the bearing of a bird or flight group within a five-degree sector to the right or left of the home direction (the vertical arrow) at

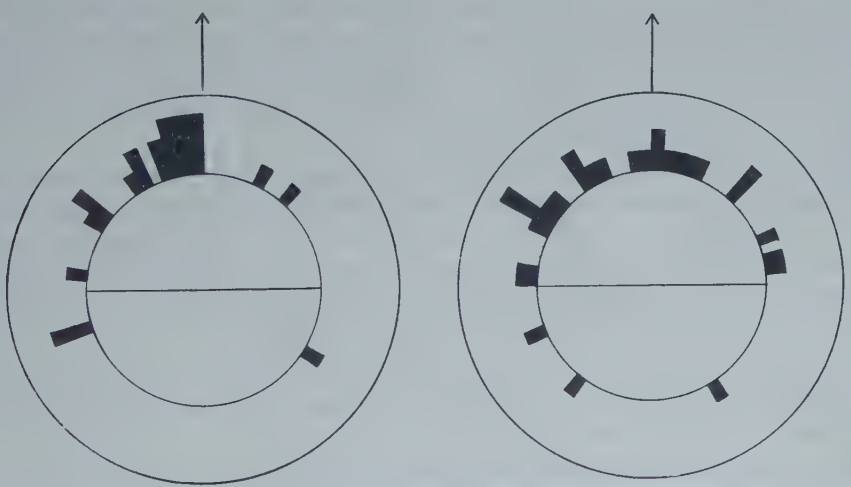

Fig. 1. Comparison of vanishing points in homing test releases for pigeons with no previous homing experience and pigeons with three previous short homing flights. Trained: av. dev. = 50·7°; S.D. = 39·0. Untrained: av. dev. = 30·0°; S.D. = 36·0.

the moment of going out of sight. The vanishing points of the untrained birds are shown in the graph on the left; those of the trained birds on the right. There is a slight suggestion of more accurate homeward orientation in the untrained pigeons, but the difference is not statistically significant.

The homeward tendency shown by each group, however, is statistically significant. For a convenient basis of evaluation, the number of vanishing points lying within the half of the circle toward home may be compared with the number in the opposite half, which two numbers are expected to be equal in a random distribution. For the untrained group a total of 20 vanishing points were toward home and 3 in the opposite direction, while the trained group gave 25 toward and 3 away from home. There is a significant departure from expectation in each case. For the untrained birds, the χ^2 value is 12·6 with one degree of freedom ($P = 0{\cdot}0004$), and for the trained birds the χ^2 is 17·3 ($P = 0{\cdot}00003$).

Homing success

Previous investigations had clearly indicated that birds with preliminary short-distance homing releases should do well in returning to the loft on their first long-distance flight (Kramer, 1953; Kramer & St Paul, 1952; Pratt, 1953). The new and more interesting question in the present investigation was, therefore, that of how the trained and untrained birds would compare in homing success. Matthews's finding of a much lower homing success in untrained birds had, as mentioned above, not yet been published.

The general homing results showed no appreciable difference between the two groups. Taking the three experiments combined, twelve out of twenty-four or 50% of the untrained birds homed by the second day following the release, while seventeen out of thirty-one or 55% of the trained birds were back in the same time. One untrained bird homed after 10 days and one trained bird, after 24 days. The average speed was slow. Matthews (1951) found that the average speed for birds home on the day of release was approximately 22 m.p.h. I had only seven birds back on the day of release, a lower percentage than Matthews reported, and the average speed of these seven was only slightly above 14 m.p.h.

Since birds of both groups were released on the same day, a more detailed analysis is required to find out whether the untrained birds were able to home independently of the trained birds. Only in the case that some of the untrained birds kept the lead they were given at the release point in being set free ahead of the trained birds and were the first to arrive at the loft could it be stated definitely that they had not been guided or encouraged over the last part of the journey.

Only three birds homed in the $\text{Bishopville}_{\text{II}}$ experiment, and they had all had previous short homing flights. There were only three untrained birds in this experiment out of a total of thirteen.

The second experiment, $\text{Hoffman}_{\text{I}}$, also failed to give a clear-cut answer to this question of whether the untrained birds would home unassisted. There were three untrained and three trained birds in the younger group of Duke stock. Two birds, one with and one without previous homing experience, reached the loft together on the day of release. The untrained bird took 6 hr. and the trained one, $5\frac{1}{2}$ hr., to cover the 75 miles. In the case of the older birds from the Cranford loft, the three untrained birds that were released ahead of the others all reached the loft first and at the same time. The time for the fastest bird in the group was approximately 3 hr.

However, 5 min. after the three untrained birds entered the loft, the first trained bird reached home after a flight of 2 hr. 20 min. The time interval between these arrivals was too short to rule out the possibility that the four arrived in a group, as the trained bird may have rested before entering the loft.

The order of releases in the third experiment, as in the first one, was arranged so that all the untrained birds had departed before any of the trained birds were set free. Fifteen untrained birds were used in this experiment, and this larger number meant that it would take longer to get them all away. The first birds released would therefore have appreciably more time to get ahead of the trained birds, and this fact would increase further their likelihood of arriving home without being overtaken. To give even the last untrained birds released a more distinct advantage, a delay of an hour was interposed before the first trained birds were set free.

These measures were successful. The first two birds to reach home, one at 5.00 p.m. on the day of release and the other at 5.40 p.m., were both of the untrained group. The third bird, a trained one, did not reach the loft until 6.30 p.m., 50 min. behind the second untrained bird. These three were the only birds that reached home on the day of release. Most of the pigeons arriving on the following days came in singly, so the independence of homing success was apparent even among the late arrivals.

Results of subsequent tests with untrained birds

Since this paper was first written, Dr R. H. Thouless and I have collaborated in a further series of experiments bearing upon a different aspect of the homing problem. In our experiments some releases were, as a matter of convenience, made entirely with untrained birds. These releases provide additional evidence on the homing behaviour of untrained pigeons, particularly on the question of their homing success when there was no possibility of being led by trained birds. The results from our tests with first-flight birds are summarized below. The releases are listed in the order of increasing distances rather than chronologically. On each occasion, single bird releases were made, and the general procedure was the same as that described for the previous experiments. The birds were 4–6 months of age.

(1) Lick Stone, 25 Oct. 1953. Three birds. Loft: White Pine, Tennessee, 40 miles away across high mountains, 348° bearing. Weather: clear, calm, and mild. Vanishing points: 325°, 10°, 47°. The releases were made about 2 hr. before sunset. Two of the birds reached home on the following day and the third a day later.

(2) Warren, 14 Nov. 1953. Five birds. Loft: Hillsboro, 65 miles, 244° bearing. Weather: clear, calm, and mild. Vanishing points: 227°, 240°, 250°, 300°, 330°. One bird homed in 4 hr. 49 min. on the day of release, a second on the forenoon of the next day, and a third on the second day following the release. Two birds were lost.

(3) Medoc, 14 Nov. 1953 (second release point used on this day). Four birds. Loft: Hillsboro, 59 miles, 259° bearing. Weather: clear, calm, and mild. Vanishing points: 231°, 270°, 288°, 294°. Three birds homed on the day after release, the fourth was lost.

(4) Dugger Mt., 7 Oct. 1953. Eight birds. Loft: White Pine, 96 miles, 268° bearing. Weather: clear, wind from the north approximately 10 m.p.h., temperature 45–60° F. The vanishing points were randomly distributed (possibly due to the facts that the birds had been confined in cages for 4 days and were shipped on a round-about journey of 500 miles before reaching the release point). Three birds homed over mountainous country

on the second, fourth, and sixth days, one refused to fly and was recaptured at the release point, and four were lost.

(5) Lowes Grove, 1 Nov. 1953. Three birds. Loft: Richmond, Va., 138 miles, 36° bearing. Weather: high veil-like clouds, clearing, calm, temperature 70° F. Vanishing points: 89°, 98°, 172°. These releases were made in the middle of the afternoon, and one bird homed at 8.00 a.m. on the following day after approximately 6 hr. of daylight. (This release was not in the Thouless-Pratt series.)

(6) Dugger Mt., 7 Oct. 1953. Twenty birds. Loft: Hillsboro, 140 miles, 92° bearing. Weather: clear, wind from the north approximately 10 m.p.h., temperature 45–60° F. These birds were a second group released at Dugger Mt. on this occasion. Their loft was toward the east, opposite the home direction of the other birds released at this point as described under (4) above. The vanishing points are shown in Fig. 2. In spite of the general tendency to depart within the home half of the circle, only two birds returned to the loft, one on the tenth day and the other some time later.

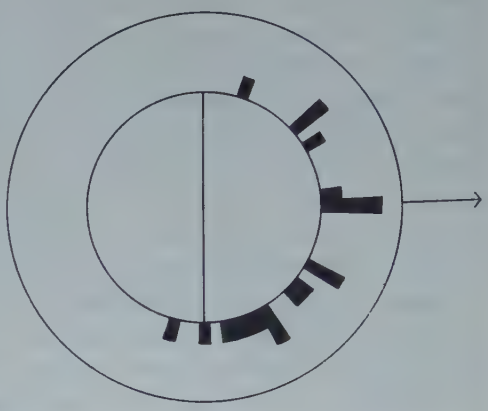

Fig. 2. Dugger Mt. releases of Hillsboro birds.

The success in regaining the loft shown in this series of releases is inversely proportional to the distance. The results suggest that 100 miles may be about as far as young pigeons of this stock may be expected to home in the North Carolina situation without previous experience with shorter flights.

In $\text{Hoffman}_{\text{I}}$ and $\text{Hoffman}_{\text{II}}$ some special comparisons were made to test possible factors affecting either flight orientation at the release point or homing success, or both. These special conditions and results follow.

Does the height of the release point influence the vanishing time?

A number of investigators have called attention to a tendency of pigeons to fly about near the release point before moving off in a chosen direction. This so-called circling behaviour was so prevalent in the departure flights that it was assumed to be an essential characteristic of the beginning stage of homing flight. Hitchcock (1952), for example, calls it 'orientation flight', and Matthews (1953*a*), in his sun navigation hypothesis of homing, supposes that some delaying action is necessary to allow the pigeon to 'fix' its position on the basis of the apparent motion of the sun.

Kramer & St Paul (1952), on the other hand, reported immediate departures quite accurately oriented toward home, with no circling. Indeed, this was one of the most surprising aspects of their results.

It occurred to me that the height of the release above the ground or the surrounding countryside might influence the amount of circling. Both Hitchcock and Matthews presumably released from the ground in relatively flat country in most instances (New England airports and English terrain, respectively), though informa-

tion regarding each point is not given. Kramer and his co-workers, on the other hand, have used a castle tower on a hilltop as their main release point. They observed that their birds lost altitude in taking off. These observations suggested that birds released on the ground may circle to gain altitude before they show any homeward orientation. It is conceivable that the use of a sufficiently high release point may cancel out the need for any unoriented flight near the release point.

To put this hypothesis to an experimental test, the birds of $\text{Hoffman}_{\text{I}}$ and $\text{Hoffman}_{\text{II}}$ were released with the crates being alternately at the top of a 100 ft. tower and on the ground. The first experiment gave rather strong evidence for more immediate departures by the birds released from the top of the tower. The average vanishing time of the tower birds was 2·9 min., while that for the ground birds was 5·3 min. The number of flights is small for statistical analysis. Nevertheless, the difference between the two distributions was tested by Student's t test. The value of t was found to be 1·76 (13 degrees of freedom) with $P = 0{\cdot}05$.

This was encouraging, and $\text{Hoffman}_{\text{II}}$ afforded an opportunity to make a further comparison of ground and tower releases. In this experiment there was not so much difference in the average vanishing times of the two groups, but the difference was in the same sense as in the previous experiment. The tower birds averaged 3·6 min. to vanish, while the ground birds took an average time of 4·6 min. This difference gives a t of 1·32 (27 D.F.), with $P = 0{\cdot}1$.

As an estimate of the significance of the difference between all ground and tower releases, the t of the difference between these two groups for both Hoffman experiments combined was obtained. This analysis gave $t = 2{\cdot}28$ (42 D.F.), with $P = 0{\cdot}023$. The indications regarding the height of the release point upon the immediacy of departure are therefore strong enough to justify further tests along these lines as opportunities for them are found. It was observed that in these experiments the birds flew at different heights on the 2 days. The $\text{Hoffman}_{\text{I}}$ birds flew above the height of the tower, possibly at 200 or 300 ft. Those of $\text{Hoffman}_{\text{II}}$, on the other hand, flew much nearer the ground. Other investigators have observed this tendency for pigeons to fly at different heights on different days. It is conceivable, therefore, that $\text{Hoffman}_{\text{II}}$ happened to fall on one of those days on which the factors tending to cause low flight predominated. If so, the fact of being released from the ground may have resulted in relatively less delay in comparison with the tower birds than had been observed with the high-flying departures of $\text{Hoffman}_{\text{I}}$.

The question of how quickly after being released the birds are able to show homeward orientation has become one of fundamental importance for various hypotheses of homing ability. If the delaying, circling flight at the release point should be found to have no direct bearing upon homing orientation, these hypotheses would be weakened, if not disproved, by this discovery. In this event, the differences in behaviour during the first few minutes of flight as recorded by different investigators would remain to be explained, but the fact that pigeons frequently circle before starting home would then appear to be only indirectly related to homing orientation.

An apparent stock difference in homing performance

The Hoffman$_{I}$ birds fell into two age groups, each composed of birds hatched from different homing stock. The younger, Duke birds were hatched from stock obtained from several sources. No special effort was made to select birds of outstanding homing ability. The birds in the older age group were from the loft of Mr E. B. Cranford of Durham, North Carolina. This loft was started with selected racing stock. Six weeks before Hoffman$_{I}$, Mr Cranford turned his loft and all his birds over to me. The nine birds had no homing experience except for one that I released 40 miles to the E.N.E. 6 months previously. This bird was put in the trained group for the experiment.

In the departure flights the vanishing points of the younger birds were more widely scattered than were those of the older group, though both groups showed departures entirely within the home half of the circle. However, the homing returns showed a striking difference between the two groups. Only two of the six birds of Duke stock reached home, whereas eight of the nine Cranford birds did so. The probability of finding a difference in homing success at least as great as this, calculated by the exact method as shown in Table 1, is suggestive ($P=0{\cdot}047$), though the number of pigeons is too small to indicate more than the need for further study of stock and age differences.

For Hoffman$_{II}$ an opportunity was sought for distinguishing between these two factors. One group used in this experiment consisted of fifteen birds of Duke stock. Eleven of them were 7–8 months of age, the other four, $4\frac{1}{2}$ months. The other group consisted of fourteen birds hatched in the racing loft of Mr R. R. Grundy of Richmond and imported into the Duke loft at 28 days. These birds were $4\frac{1}{2}$–5 months of age at the time of Hoffman$_{II}$. In the second experiment there was thus a difference in stock comparable to that of the earlier experiment, but the age relationship was reversed. If age was the major factor in the results of the first experiment, the older birds of Duke stock should show superior homing performance in Hoffman$_{II}$. On the other hand, if the hereditary factor was more important, the Grundy stock should do better.

As in the previous experiment, the Hoffman$_{II}$ observations at the release point did not show any clear-cut difference between the two stocks. The indications are therefore that within the ranges tested age and hereditary differences have little effect upon homeward orientation at the point of release.

The homing returns of Hoffman$_{II}$ were, however, again quite different in the two groups, and the direction of difference pointed to the hereditary factor as the important one. Thirteen out of fourteen of the Grundy birds reached home by the early forenoon of the second day following the release. By contrast, only seven out of fifteen Duke birds homed successfully, and one of these was not identified in the loft until the ninth day following the release. By the exact method of calculation (see Table 1) this difference is statistically significant ($P=0{\cdot}01$).

The birds carried message bands requesting information if they were found. The single stray of the Grundy group was reported as having overflown the loft by

approximately 40 miles. Three birds of the Duke stock were reported: two with wing injuries within 10 miles of the release point, and the third about 15 miles short of the loft. If the younger age of the Grundy stock was a handicap, it was more than offset by the hereditary factor or factors affecting success in regaining the loft.

Table 1. *Homing success in relation to stock*

Hoffman$_{I}$:

	Stock: Duke	Stock: Cranford	
Homed	2	8	10
Lost	4	1	5
	6	9	15

$$P=\frac{10!\,5!\,6!\,9!}{15!\,2!\,8!\,4!\,1!}+\frac{10!\,5!\,6!\,9!}{15!\,1!\,9!\,5!\,0!}=0{\cdot}047$$

Hoffman$_{II}$:

	Stock: Duke	Stock: Grundy	
Homed	7	13	20
Lost	8	1	9
	15	14	29

$$P=\frac{20!\,9!\,15!\,14!}{29!\,7!\,13!\,8!\,1!}+\frac{20!\,9!\,15!\,14!}{29!\,6!\,14!\,9!\,0!}=0{\cdot}01$$

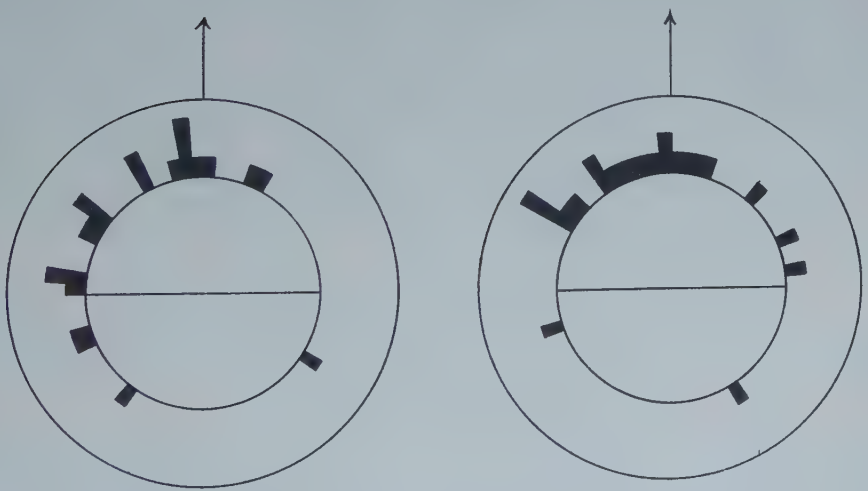

Fig. 3. Comparison of vanishing points of Duke stock and the other two stocks used in Hoffman$_{I}$. and Hoffman$_{II}$. Duke stock: av. dev.$=48{\cdot}9°$; S.D.$=50{\cdot}0$. Cranford and Grundy stock: av. dev. $40{\cdot}4°$; S.D. $35{\cdot}7$.

The difference in the vanishing points of the Duke stock in the two experiments and the other two stocks is shown in Fig. 3. The Duke pigeons (left-hand graph) show a somewhat greater spread of vanishing points, but the homeward direction of the departure flights of both groups is clearly evident.

Is homing influenced by previous experience of being transported?

It is necessary before pigeons can be given a distant release to confine them under unnatural conditions during transport. The question arises whether these special conditions affect either the release orientation or the returns. To test this point, I divided the birds used in $Hoffman_{II}$ into two groups, each one to receive a different amount of hauling experience in the preliminary period of treatment. These groups cut across the groups involving differences in training and stock. (1) The birds of one group went through the treatment period with the smallest possible amount of transportation. Half of them were untrained birds that were not placed in the trunk of the car until the time for the journey to Hoffman. The others were trained birds for which the hauling was limited to the shortest possible time on the three short-distance releases. (2) The other treatment was one that simulated the journey to Hoffman 3 times during the treatment period. The birds were kept in the trunk of the car while it was used in normal daily driving, with intermittent starts and stops, for a period roughly equivalent to that required for the final journey. For the trained birds in this group, the hauling period ended at the designated release point for one of the preliminary homing flights, while for the untrained birds in this group, it ended at the loft.

There were no differences in homing behaviour related to the amount of preliminary treatment.

DISCUSSION

The results of the present investigation confirm the recent findings of other experiments that pigeons have an ability to turn toward home when they are released in strange territory out of sight of familiar landmarks. The interpretation of the vanishing points as evidence of directed homing may be questioned on the ground that only two release points were used. The departure flights of pigeons may be influenced by local factors at the release point that are not yet sufficiently known to be taken fully into account. Objects on the horizon resembling those seen from the loft (such as water tanks); nearby cities, hills, and bodies of water; wind and weather conditions: these are only some of the factors that might conceivably influence departures and cause the flights to be grouped in one sector. If so, the separate releases at one point would not be independent and it might be necessary to take the average direction from each place as one observation and to rely on the use of a number of release points selected at random.*

The number of separate places from which releases have now been made, including those reported by Hitchcock, Matthews, and Kramer and his co-workers, is at least twenty, and releases from new locations are being made at a rapid rate. The departures have been directed toward home at almost all the places. It now seems very unlikely, therefore, that local features at any particular point that would escape the notice of the experimenter will be found to play a very important part in determining the direction of the departure flights. With only this slight reservation,

* Another procedure, suggested by Dr Kramer, is to use birds with lofts in different directions from the release point.

the results reported here support the conclusion that the direction of the home loft was the factor which caused the birds to vanish in that half of the circle.

The main question is, of course, how the birds are able to show this homeward orientation upon release. (Once they are pointed in the right direction one can conceive of relatively simple ways in which they can fly in a straight line, though the problem of how the orientation is maintained is also one for experimental investigation.) The hypothesis that the birds depend on random searching until they come upon familiar landmarks is disproved by the fact that the homeward orientation can be observed at the release point. The present findings merely contribute to the general knowledge of the factors influencing homing. Some of the results are only of suggestive value, but (if they are confirmed by further investigations) they may have some bearing upon later efforts to solve the basic homing problem.

The three tests in this report are the first to make a comparison between birds with and without previous homing experience released on the same day. Matthews (1953*b*) compared the homing behaviour of untrained birds with the results of tests with trained birds from different days. In one respect (release orientation), the present results confirm Matthews's findings, but in another (homing success) they differ sharply from his data. In both investigations birds not previously released out of sight of the loft and transported under strictly controlled conditions for long-distance releases started promptly toward home. However, the untrained pigeons released by Matthews were much less successful than those of the present investigation in returning to the loft. The difference in homing success is highly significant, but I cannot explain it. Four factors that may be kept in mind are stock, method of handling the birds, mixing of trained and untrained birds on the homing flight, and differences in geographical and weather conditions. Discussion of these may be postponed until further tests show whether the difference persists.

Table 2. *Homing success of untrained pigeons*

	Matthews's study	Present study	
Homed	1	13	14
Lost	38	12	50
	39	25	64

χ^2 (with Yates's correction) = 19·0 (1 D.F.); P = 0·00002

Until recently, there was widespread doubt among scientists regarding whether pigeons had any genuine homing ability. Even after Matthews concluded that they had, he expressed the opinion that their ability to home depended upon previous training. It now seems clear, both from his own later work and from this study, that homing orientation may occur as a form of *unlearned* behaviour, and it is therefore much more closely related to the forms of behaviour generally classed as innate or instinctive than it was commonly supposed to be. What the significance of this fact may be for furthering scientific understanding of homing ability is a question upon which speculation may well wait until further experimental facts are available.

If the suggestion regarding the effect of the height of the release point upon how quickly the pigeon shows its homeward orientation is confirmed, this discovery would make it easier to distinguish among various hypotheses of homing that have been offered. Some hypotheses require a short period of circling flight while the bird is receiving and testing the stimuli necessary for a sensory discrimination of the way toward home. The present findings suggest that these hypotheses may be based upon a misinterpretation of the circling near the release point. One way to test any principle which assumes that the bird is momentarily lost is to release birds under conditions enabling them to orient toward home without delay. Making releases from high points may be one means of helping to get quick departures.

The question of the relation of pigeon stock and homing performance likewise has both practical and theoretical significance. Matthews (1951) suggested that there were differences in his experimental results related to different strains of pigeons. Most investigators have assumed that good homing stock is important for success in homing research. This report presents results which suggest that hereditary differences chiefly influence the success in reaching home. This finding needs to be tested with stocks having a more clearly defined difference in heredity.

SUMMARY

1. Three different groups of pigeons were given homing releases, the first at 143 miles and the other two at 75 miles. Approximately half of the birds (untrained group) had made no homing flight before they were released in the long-distance test. The others (trained group) had been released 3 times in different directions at 10 miles from the loft in one experiment and at 2–3 miles in the other two.

2. Both the trained and untrained birds showed a statistically significant tendency to vanish from sight at the release point in the homeward half of the circle, which confirms Matthews's results with untrained pigeons. There was no appreciable difference in homing success and speed between these two groups. More than 50% of each group homed, while Matthews had only one out of thirty-nine untrained birds return to the loft.

3. The untrained birds were released ahead of the trained birds in each experiment. In the third experiment, two untrained birds homed, separately, without being overtaken by any trained birds. Thus these two birds showed clearly that pigeons without previous homing experience could and would complete a long-distance flight to the loft unassisted. Subsequent tests in which only untrained birds were released have confirmed this finding.

4. Birds differing in both age and stock were compared in the second and third experiments. Within the range of 4–9 months, age was not found to be a factor in homing behaviour. Stock or the hereditary factor, however, was found to be related to success in regaining the loft, though it did not appear to influence the accuracy of orientation shown by the departure flights.

5. There is a suggestion that the height of the release point influences the immediacy of departure. Birds set free from the top of a 100 ft. tower vanished more quickly than pigeons released on the ground.

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THE EXCRETION AND STORAGE OF AMMONIA BY THE AQUATIC LARVA OF *SIALIS LUTARIA* (NEUROPTERA)

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INTRODUCTION

Delaunay (1931), in a review of the invertebrates, showed that an excellent correlation existed between the nature of the major nitrogenous component of the excreta and the nature of the environment, aquatic or terrestrial, in which an animal lived. Ammonia was shown to predominate in the excreta of aquatic species, urea or uric acid in the excreta of semi-terrestrial or terrestrial species. Delaunay put forward the view that the synthesis by these terrestrial forms of more complex molecules from ammonia was essentially a detoxication mechanism necessitated by a restricted water supply.

Although the insects are primarily a terrestrial group, representatives of a number of orders have become aquatic in one or more stages of their life histories. It is well known that those terrestrial species which have been examined, with the notable exception of blowfly larvae, excrete the bulk of their nitrogen in the form of uric acid (Wigglesworth, 1950). The possibility, however, that aquatic species might have reverted to ammonotelism does not seem to have been examined.

Preliminary tests were carried out on the excreta of a variety of aquatic insects. In all cases ammonia was found to be the major nitrogenous excretory product. An investigation into various aspects of the metabolism, toxicity and excretion of ammonia was then undertaken on the aquatic larva of *Sialis lutaria*. It is the purpose of the present communication to present some observations on the excretion and storage of ammonia in this species.

MATERIAL AND METHODS

Larvae of *Sialis* were obtained in abundance from ponds in the vicinity of Newcastle. After collection they were starved for a period of at least 1 week in slowly running tap water. During this period the bulk of the food residues in the gut was eliminated.

Ammonia was estimated by the ultra-micro diffusion method described by Shaw & Beadle (1949). Using N/100 acid and alkali, quantities of ammonia were estimated ranging from 0·1 to 1·0 μg. N with a standard deviation of 0·005 μg. N.

Total nitrogen was estimated by the ultra-micro Kjeldahl method of Shaw & Beadle (1949). Using N/20 acid and alkali, quantities of nitrogen, ranging from 0·5 to 5·0 μg., were estimated with a standard deviation of 0·04 μg. N.

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The procedure adopted for the extraction of ammonia from whole animals was as follows. A single larva was weighed then ground up for 3–4 min. in the bottom of a Pyrex centrifuge tube (1 × 7 cm.) containing glass powder and 0·5 or 1·0 ml. of a 10% solution of trichloroacetic acid. The tube was then centrifuged, and samples of the supernatant taken for analysis 30 min. later. The total volume of supernatant was obtained by combining the original volume of trichloroacetic acid with the water content of the larva. Desiccation experiments showed that the amount of water contained in a single larva was in the region of 80% of the original body weight. The liberation of ammonia which occurs when tissues are ground in water was found to be completely inhibited by trichloroacetic acid. Furthermore, suitable experiments showed that the extraction of ammonia was complete. The precaution of emptying the hindgut was always taken before extracting ammonia.

Larvae were weighed on a 500 mg. torsion balance.

For purposes of collecting either foregut fluid or hindgut fluid an animal was first narcotized in water through which CO_2 was gently bubbled (Beadle & Shaw, 1950) and then dried on filter-paper. The foregut fluid, generally brown in colour, was removed by inserting a glass capillary into the foregut through the mouth. In general, a sufficient amount was obtained to enable duplicate ammonia estimations to be made, each requiring in the order of 0·3 μl. The larva could be induced to eject the clear, colourless hindgut fluid by gently stimulating the hind region with a glass capillary when signs of recovery started to appear. As the fluid appeared, generally as a discrete drop, it was collected in the capillary. Generally sufficient was obtained to enable duplicate ammonia estimations to be made, each requiring in the order of 0·15–0·30 μl. Contamination of the clear midgut fluid with brown foregut fluid was never observed to occur.

The method of Beadle & Shaw (1950), with slight modification, was used for collecting haemolymph. First, the pronotum was made hydrofuge by application of wax made molten with a heated needle. A puncture was then made and the haemolymph extruded by gently squeezing the abdomen between forefinger and thumb. As the haemolymph appeared it was collected in a glass capillary. The amount of haemolymph removed generally varied from 10 to 20% of the original body weight.

THE EXCRETION OF AMMONIA

(*a*) *Ammonia in the excreta*

In order to establish the extent to which nitrogen was being excreted in the form of ammonia, duplicate total N and ammonia N analyses, each requiring 58 μl., were carried out on the water (0·5–0·75 ml.) in which a larva had been kept for a period of 2 days. The glass tube (2 × 7 cm.) containing water and larva was corked during the experimental period. Control experiments showed that there was no loss of ammonia during this period. Five experiments were performed with larvae varying in weight from 40 to 83 mg. The total N excreted averaged 11·0 (5·5–17·5) μg./100 mg. wet weight/24 hr. Of the total N excreted 63–97% (av. 86%) was found to be in the form of ammonia. The figures in brackets refer to the lowest and highest values respectively.

Confirmation that the volatile base estimated by diffusion analysis was in fact ammonia was obtained by comparing results obtained by diffusion analysis with results obtained by the well known Nessler colorimetric method. Six samples of excreta were subjected to analysis. The differences between the results obtained by the two methods were found, when subjected to the '*t*' test for significance, to be no greater than those which would be expected by chance. Trimethylamine, which has been found in the excreta of the marine teleost *Lophius piscatorius* (Grollman, 1929), is perhaps the only other volatile base likely to be met with in the excreta. In low concentration, however, trimethylamine develops neither colour nor precipitate with Nessler's reagent. The similarity of the results obtained by the two methods indicates, therefore, that the volatile nitrogenous base which is excreted is in fact ammonia.

(*b*) *The site of ammonia excretion*

The intestine of *Sialis* consists of a capacious foregut (generally containing a brown fluid), a very narrow midgut, and a hindgut, capable of distension, in which a clear colourless fluid accumulates, to be expelled periodically through the anus. The Malpighian tubules enter the intestine at the junction of midgut and hindgut. There seems little reason to doubt, although no experiments have been carried out to test this assumption, that the hindgut fluid is in fact urine having origin in the Malpighian tubules. In support of this contention, contamination of the clear, colourless hindgut fluid with brown foregut material has never been observed to occur.

Ammonia was never found in the foregut fluid (results of five separate estimations). On the other hand, the concentration of ammonia in the hindgut fluid was found to be very high—the results of five separate estimations, performed on the fluid removed from different larvae, ranged from 97 to 159 mg. ammonia N/100 ml. (average 136 mg. N/100 ml.). These results suggest that ammonia is being excreted by the Malpighian tubules. No attempt has yet been made, however, to establish the concentration of ammonia in the tubules.

Although it seemed unlikely, on the basis of the results just mentioned, that any excretion of ammonia across the general body surface was in fact occurring, the possibility was nevertheless checked. To test this possibility nine animals were treated in the following way. To prevent any elimination of fluid from the gut the mouth of each animal was blocked with wax and the region of the body just in front of the anus ligatured. Each animal was placed in 3 ml. of distilled water. At the end of 42 hr. the ammonia content of the water was determined. Ammonia was found to be absent in all but two cases, and then the quantity of ammonia found—less than one-tenth of that normally excreted by animals of similar weight—was very small, probably due to slight leakage of the highly ammoniacal hindgut fluid. It can be concluded, therefore, that ammonia is not excreted across the general body surface.

THE STORAGE OF AMMONIA

(*a*) *The ammonia content of tissue fluids of normal larvae*

Estimations have been made to establish to what extent the tissue fluids are being maintained ammonia free.

The total ammonia content of thirteen larvae was determined. In all cases the ammonia content was found to be very low, varying from 0·5 to 2·2 μg. N/100 mg. wet weight of tissue (average 1·0 μg. N).

Separate estimations were performed to determine the concentration of ammonia in the haemolymph. Analyses (duplicate) made within 1 min. after extraction and then 10 min. later (using 1·8 μl. for a single analysis) failed to detect any liberation of ammonia in the shed fluid. It is presumed, therefore, that the concentration of ammonia found in the shed haemolymph represents the actual concentration found *in vivo*.

Accurate measurements of the concentration of ammonia in the haemolymph were obtained by making duplicate estimations, each requiring 14 or 28 μl., on the combined fluid removed from a number of larvae. Care was taken to ensure that the whole process from extraction to analysis took no more than 10 min. Estimations were made on five such samples. The results obtained, varying from 0·37 to 0·76 mg. ammonia N/100 ml. (average 0·50 mg. N/100 ml.), demonstrated that ammonia was not completely removed from the haemolymph.

(*b*) *The effect of preventing excretion on the ammonia content of the body*

In the hope of obtaining some answer to the question of whether larval tissues are capable of storing ammonia the following experiment was performed.

An animal was narcotized with CO_2 and then dried on filter-paper. The mouth was blocked (to prevent drinking) by the application of molten wax and a ligature applied round the abdomen (the site of ligature varied for reasons given below). The region posterior to the ligature was now cut off and the wound waxed over. The preparation was kept in an approximate isotonic solution of dextrose (6·2 g./100 ml. changed every day) for 5 days at room temperature. At the end of this period analyses were performed to establish the concentration of ammonia in the haemolymph and the total ammonia content of the body portion. The volume of haemolymph extracted was determined by weighing the preparation before and after extraction. The total ammonia content of the extruded haemolymph was combined with the total ammonia content of the body portion to give the true ammonia content of the body portion.

Two groups, each of six animals, were prepared. In the first group the ligature was applied between the fourth and fifth abdominal segments. This ligature lies in front of the point of entry of the Malpighian tubules into the gut. Thus there can be no question of urine passing into the gut. Animals of the second group were ligatured between the thorax and abdomen. These preparations are of interest because the Malpighian tubules do not extend further forwards than the junction between thorax and abdomen. Thus such preparations are devoid of Malpighian tubule tissue.

All preparations were alive at the end of 5 days. It has been found, however, that 'head-thorax' preparations rarely live much longer. The other type of preparation may live, and appear healthy, for as long as 3 weeks.

In the case of those preparations retaining the anterior portion of the abdomen the concentration of ammonia in the haemolymph varied from 0·0 to 0·9 mg. N/100 ml. (average 0·6 mg. N/100 ml.) and the total ammonia content of the body varied from 1·2 to 1·8 μg. N/100 mg. wet weight of tissue (average 1·4 μg. N/100 ml.).

In the case of those preparations ('head-thorax') devoid of Malpighian tubule tissue the concentration of ammonia in the haemolymph varied from 0·0 to 1·4 mg. N/100 ml. (average 0·6 mg. N/100 ml.) and the total ammonia content of the body varied from 0·6 to 1·7 μg. N/100 mg. wet weight of tissue (average 1·1 μg. N/100 mg.).

The values obtained in the case of both types of preparation are of the same order as those values obtained on normal larvae. These results would suggest, therefore, that larval tissues have the capacity to 'store' appreciable quantities of ammonia. Furthermore, although the Malpighian tubules themselves may be able to 'store' ammonia, this property is also found in non-Malpighian tubule tissue. The alternative possibility that deamination may have stopped does not seem feasible.

On the basis that the daily ammonia output of larvae averages 10 μg. N/100 mg. wet weight/24 hr. during starvation it can be calculated that the experimental larvae have 'stored' in the region of 50 μg. N/100 mg. wet weight of tissue in 5 days.

(*c*) *The disappearance of ammonia in larval tissue*

Support for the view that larval tissues are capable of 'storing' ammonia was obtained in the following way. The mouth of a larva was blocked with wax to prevent drinking and then the ammonia content raised by immersion for some time in a 30 mg. N/100 ml. solution of ammonia. This method of raising the ammonia content is discussed in greater detail below. A ligature was now made between the thorax and abdomen. Haemolymph was removed from the abdomen and the concentration of ammonia determined. The abdomen was now cut off and discarded. After waxing the site of amputation the anterior portion ('head-thorax') was placed in 1·0 ml. N/1000 HCl (to trap any ammonia which diffused out) for a period of 7–8 hr. At the end of this period both the ammonia content of the HCl and the anterior portion of the larva were determined and the results combined. Comparison was now made with the amount of ammonia calculated to be present in the anterior portion when first placed in dilute acid. This calculation was made on the basis of previous experiments which involved determination of the concentration of ammonia in the haemolymph removed from the abdomen followed by almost simultaneous determination of the total ammonia content of the anterior body portion. The results obtained (Table 1) show, as might be expected, that the total ammonia content of the anterior body portion is closely related to the concentration of ammonia in the haemolymph. On the average, the figure expressing the total ammonia content of the anterior body portion (μg. N/100 mg. wet weight) was found to be 10% greater than the figure obtained for expressing the concentration of ammonia in the haemolymph (mg. ammonia N/100 ml.).

Table 1. *Results of experiment to determine the relationship between the concentration of ammonia in the haemolymph and the total ammonia content of the anterior portion (head-thorax) of larvae of* Sialis

Exp. no.	1	2	3	4	5	6	7
Concentration of ammonia in haemolymph (mg. NH_3-N/100 ml.)	3·2	4·7	6·0	6·8	7·3	7·6	8·0
Weight of head-thorax (mg.)	43	48	55	52	39	33	47
Ammonia content of head-thorax (μg. NH_3-N/100 mg.)	2·4	4·0	7·0	7·4	6·4	10·0	10·0

Exp. no.	8	9	10	11	12	13	Av.
Concentration of ammonia in haemolymph (mg. NH_3-N/100 ml.)	11·5	11·5	12·0	14·4	15·5	17·0	9·7
Weight of head-thorax (mg.)	51	52	40	31	42	40	—
Ammonia content of head-thorax (μg. NH_3-N/100 mg.)	11·2	13·4	13·0	16·0	16·0	22·0	10·7

Table 2. *Results of experiment comparing the ammonia recovered from the head-thorax at the end of 7–8 hr. immersion in* N/1000 HCl *with that calculated to be present before immersion*

Time of events	Exp. no.	1	2	3	4	5	6	Av.
Before placing in N/1000 HCl	Concentration of ammonia in haemolymph (mg. NH_3-N/100 ml.)	7·2	5·0	5·3	9·2	20·8	15·0	—
	Weight of head-thorax (mg.)	56	52	44	36	45	65	—
	Ammonia content of head-thorax (μg. NH_3-N/100 mg. calculated)	7·9	5·5	5·8	10·1	22·8	16·5	—
After 7–8 hr. in N/1000 HCl	Ammonia content of head-thorax (μg. NH_3-N/100 mg.)	0·9	0·6	0·4	0·9	0·2	1·6	—
	Ammonia content of HCl (μg. NH_3-N)	0·4	0·2	0·1	0·4	2·8	0·6	—
	Combined results for ammonia content of head-thorax and HCl (μg. NH_3-N/100 mg.)	2·2	1·5	1·1	3·5	6·6	3·4	—
	% ammonia recovered	28	27	19	35	25	21	26

The experiment was performed on six larvae (Table 2). In order to confirm the results obtained the same experiment was performed on another six larvae, but instead of determining the total ammonia content of the anterior body portion at the end of 7–8 hr. the concentration of ammonia in the haemolymph was determined. Comparison was then made with the concentration which was present after immersion in the ammonia solution. The results are presented in Table 3.

The preparations remained alive and active during the experimental period.

The results obtained show that a mechanism exists in the tissues of larvae of *Sialis* for removing ammonia in chemical combination. Thus the ammonia recovered at the end of 7–8 hr. averaged only 26% of that calculated to be present after immersion in the ammonia solution.

Table 3. *Results of experiments comparing the ammonia recovered from the head-thorax (calculated) at the end of 7–8 hr. immersion in* N/1000 HCl *with that calculated to be present before immersion*

Time of events	Exp. no.	1	2	3	4	5	6	Av.
Before placing in N/1000 HCl	Concentration of ammonia in haemolymph (mg. NH_3-N/100 ml.)	5·8	8·2	5·6	14·8	12·6	11·8	—
	Weight of head-thorax (mg.)	42	48	43	52	37	50	—
	Ammonia content of head-thorax (μg. NH_3-N/100 mg. calculated)	6·4	9·0	6·2	16·3	13·9	13·0	—
After 7–8 hr. in N/1000 HCl	Concentration of ammonia in haemolymph (mg. NH_3-N/100 ml.)	1·1	3·0	1·8	3·1	2·2	1·2	—
	Ammonia content of head-thorax (μg. NH_3-N/100 mg. calculated)	1·2	3·3	2·0	3·4	2·4	1·3	—
	Ammonia content of HCl (μg. NH_3-N)	0·6	0·4	0·4	0·4	0·4	0·3	—
	Combined results for ammonia content of head-thorax and HCl (μg. NH_3-N/100 mg.)	1·8	3·7	2·4	3·8	2·8	1·6	—
	% ammonia recovered	28	41	39	23	20	13	27

(*d*) *The effect of the penetration of ammonia*

The method employed for raising the concentration of ammonia in the tissue fluids warrants some comment. It is generally supposed that ammonia is a highly toxic compound. Only in the case of birds and mammals, however, has a clear-cut demonstration been obtained that ammonia is in fact highly toxic (Sumner, 1937). A concentration in the blood of 5 mg. ammonia N/100 ml. was found to be lethal. On the other hand, the haemolymph of larvae of the blowfly *Lucilia sericata* may contain as much as 20 mg. ammonia N/100 ml. in normal circumstances (Lennox, 1941). The effect of raising the concentration of ammonia in the body fluids of larvae of *Sialis* by placing the larvae in a solution of dilute ammonia has therefore been examined.

A curve for the 'penetration' of ammonia (Fig. 1) was obtained by placing a number of larvae in a 20 mg. N/100 ml. solution of ammonia (in the experiments just described a 30 mg. N/100 ml. solution was employed to increase the rate of penetration). In order to prevent the possibility that drinking might occur the mouths of the larvae were blocked with wax. At intervals up to 3 hr. larvae were removed and analyses performed to determine the concentration of ammonia in the haemolymph. The effect on the behaviour of the animal was noted.

No toxic symptoms were apparent until the concentration of ammonia in the haemolymph had been raised to a level in the region of 7·0 mg. N/100 ml. When this concentration had been reached the larvae showed a tendency to lie upside down, legs out-stretched. Normal movements were still possible, however. Progressive deterioration set in as the concentration was increased. Larvae with a concentration of ammonia in the haemolymph in the region of 15 mg. N/100 ml. remained

motionless, upside down, only showing signs of activity, in the form of twitchings of legs and abdomen, when mechanically stimulated. Larvae with a concentration of ammonia in the haemolymph in the region of 20 mg. N/100 ml. did not even respond to mechanical stimulation.

If left in the solution of ammonia death occurred. Larvae returned to water, even those with a concentration of ammonia in the haemolymph of 20 mg. N/100 ml. recovered gradually over a period of hours. Whether a complete return to normality was obtained, however, is not known.

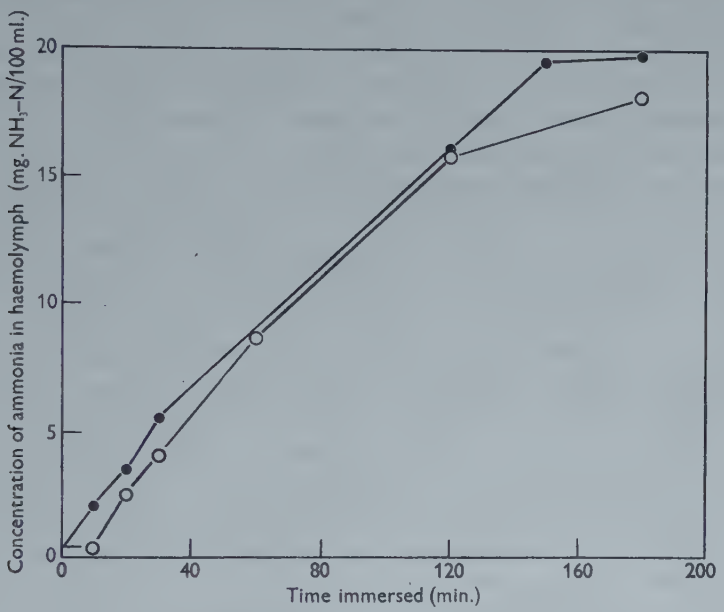

Fig. 1. Penetration of ammonia into larvae placed in a 20 mg. N/100 ml. solution of ammonia at 20° C. Symbols ○ and ● refer to two separate batches of larvae, respectively, upon which the experiment was performed.

The effect of increasing the internal concentration of ammonia on the pH of the haemolymph was also considered. The pH was determined colorimetrically using the B.D.H. Capillator. After addition of the indicator (phenol red) the end of the capillary, into which the combined haemolymph and indicator had been taken, was waxed over and the protein precipitate centrifuged down. Compensation was made for the pale yellow colour of the blood. Measurements were obtained on the haemolymph of normal larvae (the haemolymph of a single larva sufficing for one measurement) and then on the haemolymph of larvae showing marked symptoms of ammonia poisoning.

The pH of the haemolymph of seven normal larvae was found to vary from 7·0 to 7·4 (average 7·2). Values of 7·2–7·5 were obtained by Beadle & Beadle (1949). These authors measured the pH of the haemolymph of larvae of *Sialis* by means of micro-glass capillary electrodes without exposing the haemolymph to air.

The pH of the haemolymph of seven larvae with a concentration of ammonia in the haemolymph in the order of 10–15 mg. N/100 ml. was found to vary from 7·2 to 7·5 (average 7·4). Most of these values lie below the upper pH value (7·4) found in normal blood. It does not seem possible, therefore, to correlate toxic symptoms with an increase in alkalinity of the haemolymph. The possibility remains, however, that the peripheral nervous system might have been affected by local pH changes. Further investigation is therefore required before any definite conclusions can be made on the extent to which ammonia is toxic to larvae of *Sialis*. What seems certain, however, is that it is less toxic to *Sialis* than it is to mammals and birds.

DISCUSSION

It has been shown that ammonia, amounting to about 90% of the total nitrogen, is the major nitrogenous component of the excreta of larvae of *Sialis* during starvation. Since the insects are primarily a terrestrial group, and the majority of the terrestrial forms which have been examined uricotelic, it would seem probable that the larva of *Sialis* is secondarily ammonotelic. It cannot be concluded, however, on the evidence presented in this communication, that reversal is complete. Nevertheless, evidence (as yet unpublished) has been obtained which indicates that uricogenesis either does not occur or is at most trivial in normal circumstances.

The pupa and adult of *Sialis* are terrestrial. Evidence (unpublished) has been obtained that these stages in the life history are uricotelic. Thus excellent support is given to Delaunay's (1931) concept correlating the major nitrogenous component of the excreta with the nature of the environment, aquatic or terrestrial, in which an animal lives. Analyses which have been performed on the excreta of other aquatic insects give further support to this concept. Thus results have been obtained which show that ammonia, varying in amount from 70 to 90% of the total N, predominates in the excreta of nymphs of *Aeschna cyanea* (Odonata), larvae of *Phryganea striata* (Trichoptera), adults of *Acilius sulcatus* (Coleoptera) and *Dytiscus marginalis* (Coleoptera) and adults of *Notonecta glauca* (Hemiptera).

There is little reason to doubt that ammonia is being excreted by the Malpighian tubules. Further progress on the elimination side of the ammonia excretory mechanism awaits the development, however, of a procedure capable of submitting to quantitative analysis the small amount of fluid found in the tubules.

Now although 90% of the nitrogen excreted by larvae of *Sialis* is in the form of ammonia there is no accumulation in the body when excretion is prevented. It is presumed that deamination is still proceeding in such circumstances but that the resulting ammonia is being 'stored' in some way. Support was obtained for this view when it was shown that ammonia disappeared in the tissues when present in high concentration. The obvious possibility that ammonia may be undergoing synthesis into uric acid has not been discussed in this communication, but results (unpublished) which have been so far obtained indicate that this is not in fact the case. The further distinct possibility that ammonia may be undergoing storage in the form of glutamine has not yet been examined. Glutamine has in fact been found in the haemolymph of a number of insects (Pratt, 1950).

The question arises whether this mechanism plays any part in the metabolism of the normal larva. The similarity of the concentration of ammonia in the haemolymph of normal larvae and larvae prevented from excreting for a period of 5 days suggests that this may well be the case. Thus the operation of the 'storage' mechanism does not seem to require a high threshold of ammonia in the haemolymph.

It will therefore be of interest to see whether the 'storage' mechanism is reversible. If so, it may be that the 'storage' mechanism is in fact a mechanism for transporting ammonia from the tissues to the Malpighian tubules.

There is the further possibility that the real significance of the 'storage' mechanism lies in preventing accumulation of ammonia when the larva leaves the water prior to pupation and enters the marginal soil. It will therefore be of great interest to see at what precise stage during metamorphosis uricogenesis commences.

SUMMARY

1. A study has been made of the excretion and storage of ammonia by the aquatic larva of *Sialis lutaria*.

2. About 90% of the nitrogen excreted by the larva of *Sialis* during starvation was in the form of ammonia. The daily ammonia output averaged 10 μg. N/100 mg. wet weight.

3. Ammonia was found to be excreted into the hindgut, presumably via the Malpighian tubules. The concentration of ammonia in the hindgut fluid averaged 136 mg. N/100 ml.

4. Evidence was obtained that the tissue fluids are not maintained completely ammonia-free. Thus the total ammonia content of the body averaged 1·0 μg. N/100 mg. wet weight of tissue. The concentration of ammonia in the haemolymph averaged 0·50 mg. N/100 ml.

5. Evidence was obtained that the larval tissues are capable of 'storing' appreciable quantities of ammonia. Thus ammonia did not accumulate in the tissue fluids of larvae prevented from excreting for a period of 5 days. Furthermore, it was found experimentally possible to raise the concentration of ammonia in the tissue fluids, the ammonia subsequently disappearing. The possible significance of this 'storage' mechanism was discussed.

6. The method used for raising the concentration of ammonia in the tissue fluids, by immersing the larva for some time in a solution of dilute ammonia, was considered in some detail, particularly with respect to toxic effects. When the concentration of ammonia in the haemolymph had reached a level in the region of 7·0 mg. N/100 ml. toxic symptoms started to appear.

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THE CUTICULAR WATERPROOFING MECHANISM OF THE WORKER HONEY-BEE

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It is now widely accepted, following the work of Alexander, Kitchener and Briscoe (1944*a–c*), Wigglesworth (1944, 1945) and Beament (1945), that the thin layer of lipoid material which is present on or near the outer surface of insect cuticle serves to restrict the evaporation of water from the animal. Existing knowledge of the nature of this waterproofing layer has been built up from observations on the effect of dusts, heat, detergents and organic solvents on the rate of evaporation from intact cuticle and also from a study of the changes in permeability of natural and artificial membranes when coated with thin layers of lipoid material.

This paper describes experiments undertaken to obtain information on the nature of the waterproofing layer in the worker honey-bee.

PART 1. THE ACTION OF DUSTS ON WORKER HONEY-BEES

Alexander *et al.* (1944*a*) demonstrated that certain dusts were lethal to insects by virtue of their physical properties, and that this was due to their action in promoting water-loss. Wigglesworth (1944, 1945) showed that dusts can abrade the surface lipoid layer of the insect's cuticle thereby increasing the rate of water-loss through the cuticle. Beament (1945) and Alexander *et al.* (1944*c*) found that certain dusts could adsorb artificial wax films deposited on such substances as tanned gelatin which exerted little orienting force: no evidence was obtained that dusts could adsorb wax films deposited on insect epicuticle unless abrasive action augmented surface adsorption. The above-mentioned workers all indicated that dusts had no effect on dead insects. Hurst (1943, 1948) considered that the action of the dusts was due to their adsorptive properties only, and suggested that the reported 'abrasive' action resulted from the presentation of fresh surfaces of the dust to the epicuticular lipoid. He also claimed that dusts were effective in promoting cuticular water-loss in dead insects.

Preliminary experiments with bees showed that Almicide, an alumina dust, when deposited on the cuticle of both live and dead insects, effected a marked increase in the rate of water-loss through the cuticle. Although Wigglesworth (1945) showed that the rate of diffusion of water through the cuticle was a passive process not altered by the death of the insect, the action of the dusts on the dead bees was considered to be a surprising result as no mechanical abrasion of the cuticle appeared to have occurred. The following experiments were undertaken to determine the mechanism by which dusts such as Almicide effect an increase in the permeability to water of the worker bee's cuticle.

Experimental procedure

Unless stated otherwise, all the experiments were carried out on uniform groups of worker honey-bees, at least 5 days old, which had been recently killed with hydrogen sulphide. (This chemical was chosen as it is highly toxic to bees, has a rapid 'knock-down' effect, and is not expected to have any effect on lipoid material.) Twenty bees were used in each test with a similar number acting as controls. After treatment, each group of insects was placed on a small perforated zinc tray lined with filter-paper; this was weighed and put in a desiccator over calcium chloride. The trays were reweighed at intervals up to 24 hr. For convenience the results are summarized by expressing the decrease in the weight of treated bees as a multiple of the loss of the controls. It was assumed that all the additional weight lost by the treated bees was due to the evaporation of moisture; the reasons for this assumption are fully discussed by Alexander *et al.* (1944*a*).

The dusting was carried out in Exps. I–III by placing 0·5 g. of the dust (together with the bees) in a 200 ml. round-bottomed flask; the flask was slowly rotated until the insects appeared to be thoroughly coated and excess dust was then blown out of the flask with a jet of compressed air.

Experiment I. The action of Almicide on living and dead bees

Forty living bees were anaesthetized with carbon dioxide and dusted with Almicide which had been kept in a desiccator for 48 hr. Twenty of the bees were killed at once with hydrogen sulphide, the remainder being permitted to recover and live for 40 min. so that the dust might penetrate between and abrade the articulating surfaces of the cuticle. They were then killed. A further group of twenty bees was killed and used as controls. The subsequent rate of water-loss of the bees killed immediately after dusting was 3·07, and of those which had lived 4·34 times greater, than the loss in the controls. These results confirm the action of Almicide suggested by the preliminary experiments, and indicate that although the rate of water-loss was greater when abrasion of the cuticle could occur, the dust did disrupt the waterproofing layer when it came into contact with the dead insects. (During the process of dusting the hairs on the bee would act as a buffer between the cuticle and glass and prevent abrasion occurring.) A microscopic examination of both groups of dusted bees revealed that a large proportion of the dust adhering to the bee was trapped in the plumose hairs, but more dust appeared to have reached the surface of the cuticle of the bees which had lived after dusting. This alone could serve to explain the increased rate of water-loss in this group of bees.

Experiment II. The action of different dusts

Groups of bees were dusted with eleven dusts widely differing in physical properties. (Before use, each material was maintained for 24 hr. in a desiccator over calcium chloride.) The dusts varied in their adherence to the bees, but this factor was not considered to be important in a qualitative test. The results (Table 1) show that all the dusts effected an increase in the rate of water-loss of the dead bees; that the three most effective dusts were silica gel, Almicide and activated charcoal, the

common physical property of these materials being their capacity to act as powerful absorbents; that Bentonite and activated charcoal, both soft materials, were more effective than carborundum, which is hard and highly abrasive. These facts again indicate that abrasion is not an important factor in the action of these dusts in disrupting the waterproofing layers, and suggest furthermore that they may act by adsorbing the lipoid material.

Table 1. *The action of different dusts on the rate of water-loss from worker bees*

Dust	Description	Ratio of water-loss over controls
Silica gel	Powdered dust put through 300-mesh BSS sieve	7·1
Almicide	Artificially prepared alumina put through 300-mesh BSS sieve	6·0
Activated charcoal	From gas-mask, ground in mortar and put through 300-mesh BSS sieve	4·9
Slate dust	Very fine commercial sample	2·3
Fuller's earth	Commercial sample	2·2
Wyoming Bentonite	Powdered flat crystals	2·0
Carborundum	Commercial 500 grade	1·7
Pumice powder	Commercial sample	1·5
Devon clay dust	Commercial dust with high silica content	1·3
Calcium carbonate	Prepared by precipitation	1·14

Experiment III. The relationship between particle size and effectiveness

Workers with the 'inert' dust insecticides have shown that particle size was generally inversely related to effectiveness (Alexander *et al.* 1944*b*; David & Gardiner, 1950; Bartlett, 1951). To obtain data on the effect of variation in particle size on the action of dusts on the cuticle of the worker bee, two minerals were used —a range of china monodisperse fractions prepared by sedimentation and three alumina dusts of different physical characteristics. The same technique was used as in the previous experiments. Although different fractions varied in their power to adhere to the bees, the weight of dust adhering to each group did not vary more than ± 10 %. Unfortunately no measure could be obtained of the amount adhering to the cuticle itself. The results (Table 2) show that all the fractions increased cuticular water-loss. The progressive increase in effectiveness of the china clay as the particle size fell from 50 to 5 μ, confirmed the results of other workers mentioned previously, but a further decrease in size below 5 μ resulted in a decrease in effectiveness. This anomaly was explained when it was noticed on microscopic examination of the dusts that particles in the fractions 2·5 μ and less also existed as aggregates. The existence of these aggregates has been clearly demonstrated by Gregg & Hill (1953), using a similar range of kaolin fractions. In addition, it is of interest to record that whilst the fractions from 5 to 20 μ felt smooth to the touch, those under 1 μ were distinctly abrasive. Of the aluminas, the gamma grade with the smallest particles was the most effective and caused a particularly high rate of water loss. A visual inspection of the three groups of dusted bees showed that the gamma grade

had the least dense deposit, but examination under the microscope revealed that more of it had reached the cuticular surface; the other two dusts were largely trapped by the plumose hairs.

Table 2. *The relationship between particle size and effectiveness of different grades of china clay and alumina*

China clay

Size range in μ of fraction	Rate of increased water-loss over controls
54·0–30·0	1·35
30·0–20·0	1·54
20·0–10·0	2·20
10·0–5·0	2·44
5·0–2·5	2·36
2·5–1·0	2·05
1·0–0·5	2·15
Less than 0·5	1·98

Graded alumina polishing powders

Grade	Physical characteristics	Rate of increased water-loss over controls
5/20	95 % less than 5 μ 20 % less than 1 μ polyhedral or cubical crystals	1·41
3/50	95 % less than 3 μ 50 % less than 1 μ polyhedral or cubical crystals	2·81
Gamma	Cubical aggregates 1–1·5 μ having highly porous structure. True crystal size estimated 50×10^{-8} cm.	7·75

The surface of the worker bee's cuticle

In order to proceed with an examination of the action of the dust in disrupting the waterproofing mechanism, it is necessary to visualize the dust in contact with the surface of the cuticle. The presence of hairs and the apparent ridging of the cuticle itself had been noticed, and as these factors are likely to influence the deposition of dusts, their significance was considered.

The cuticle bears three types of hairs: long plumose 0·2–0·4 mm., short plumose $<0{\cdot}2$ mm., and simple setae $<0{\cdot}15$ mm. in length. The proportion of each type of hair varies on different regions of the insect. The long plumose type predominates on the thorax with an average density of 590 per mm.^{2}. Measurements from enlargements of photographs of these hairs *in situ*, and calculation based on the average hair density, suggested that dust particles greater than 2 μ in diameter will be prevented from reaching the cuticle. Tests with the china clay fractions used in Exp. III confirmed that this figure was approximately true. The abdomen has an average density of 126 long plumose hairs per mm.^{2}. Although the short simple hairs are more numerous here than on the thorax, they do not form an efficient barrier to dust

particles less than 30μ. Many areas measuring approximately 30 × 100μ were found to be devoid of hairs.

A study of the surface relief of the cuticle (Glynne Jones, Connell & Nixon, unpublished work) has shown that in the abdomen it is raised into a series of folds. Fig. 1 shows a diagrammatic section of abdominal cuticle drawn approximately to scale from sections and a phase-contrast examination of the surface (Fig. 2). In addition, the surface of each fold appears to be heavily pitted (Fig. 3).* In the thorax the folds are replaced by more distinct ridges which trace a hexagonal pattern.

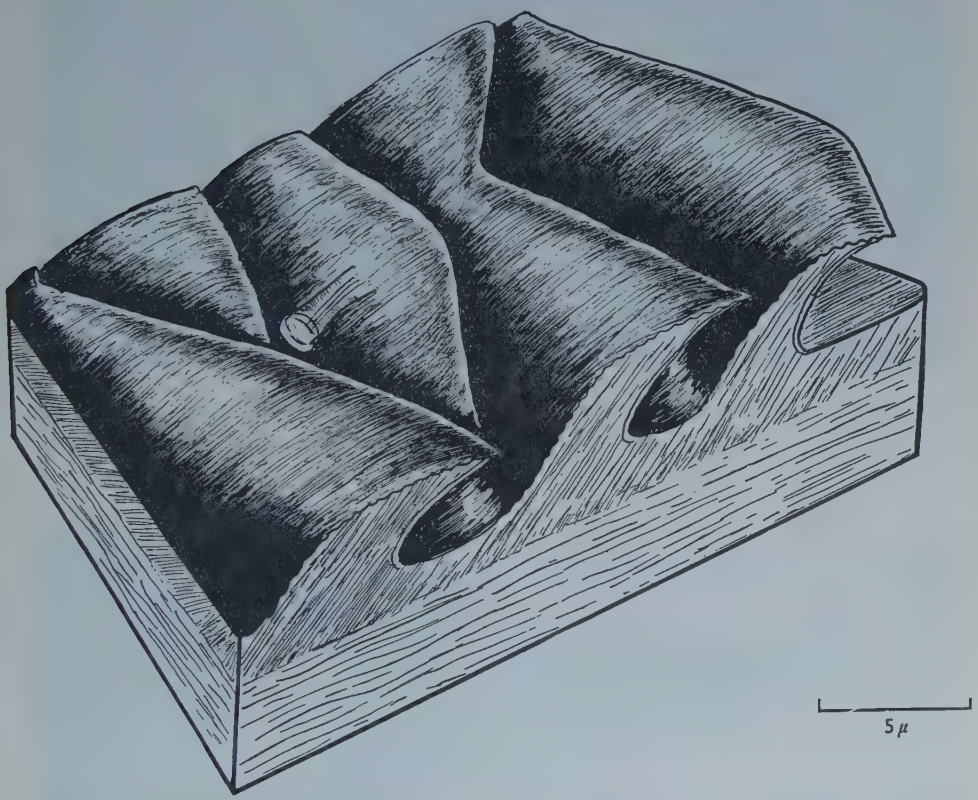

Fig. 1. Semi-diagrammatic sketch drawn to scale to show surface relief of abdominal cuticle of worker bee.

If the cuticular surface was smooth, the worker bee would have a surface area of approximately 2·2 cm.^{2}, but if the folds and pitting are taken into account the true surface area may be at least 10 times this value.

It would appear, therefore, that when a bee is dusted, as in Exps. I–III, the following considerations arise:

(*a*) A proportion of the dust applied will be held back by the plumose hairs, particularly in the thorax.

* *Note added in proof.* Further work has suggested that the large pits shown in Fig. 3 are artefacts.

Fig. 2. Phase-contrast surface view of abdominal cuticle of worker bee. × 2400.

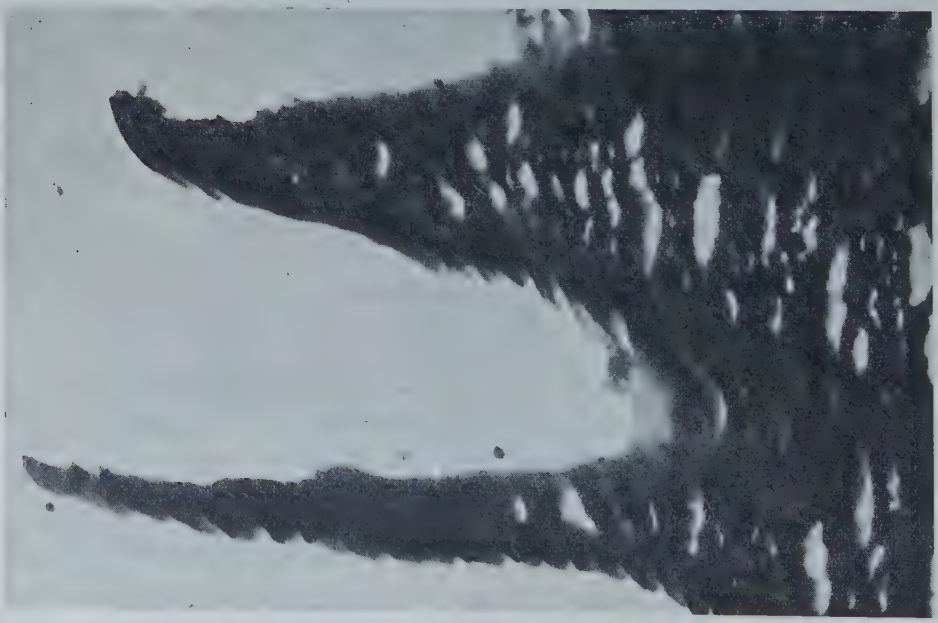

Fig. 3. Electron micrograph of transverse section of abdominal cuticle of bee showing folds. × 3680.

(*b*) Dust particles or aggregates greater than 5 μ will tend to rest on the uppermost parts of the folds and only come into contact with a relatively small proportion of the total surface area of the cuticle.

(*c*) The agitation of an insect after dusting will tend to promote the setting free of some of the particles trapped by the hairs, the settling of particles between the folds, and the breaking up of aggregates.

(*d*) When a bee is heavily dusted, the particles trapped in the plumose hairs and also resting on the folds of the cuticle may form a barrier sufficiently dense to restrict water-loss.

It thus appeared, that if the dusting technique in Exps. I–III was modified so as to prevent aggregates of large dust particles settling on the tips of the folds, thereby restricting the access of further particles to the underlying areas of the cuticle, the effectiveness of the dust would be increased as it would be brought into contact with a much larger surface area of the cuticle. Two such techniques were developed, one using an aqueous suspension of a dust, and the other, a dust cloud of very fine particles. These will now be described.

Experiment IV. Dipping bees in an aqueous suspension of alumina dust

A 1 % (w/v) suspension of the gamma grade alumina was prepared using 0·05 % (w/v) Bentonite as a suspending agent, and 0·01 % Lissapol as a wetter. This suspension was violently agitated to aid the dispersion of aggregates. Twenty recently killed bees were immersed in the suspension for 2 min. and another twenty bees were placed in water containing the same amounts of Bentonite and Lissapol for the same time, to act as controls. The superficial moisture was dried off in an air oven at 30° C. and the two groups of bees placed in a desiccator. The subsequent rate of water-loss of the treated bees was 12·2 times greater than in the controls. It is appreciated that the alumina itself is hygroscopic and that this introduces an error into the comparison of rates of water-loss. However, subsequent examination showed that the amount of alumina adhering to the cuticle was very small, probably less than 10 mg./bee, and it was estimated that the error introduced could not affect the results by more than 10 %.

The increased effectiveness of this dust when applied as an aqueous suspension suggests that the technique brings the particles into contact with a larger surface area of the cuticle than is possible with flask dusting. Furthermore, this experiment clearly shows that the dust need not abrade the cuticle to be effective. Wet dusts have been shown to be inactive (Hurst, 1948; Bartlett, 1951) and if this is the case the alumina could not exert any effect until after the bees had been placed in the desiccator and there no movement of the dust particles against the cuticle was possible.

Experiment V. Exposure of bees to a dust cloud of activated charcoal

Approximately 5 g. of finely ground activated charcoal which had been dried at 100° C. for 24 hr. and passed through a 300-mesh BSS sieve was placed in a glass tube 1 m. in length and 4 cm. in diameter which had a single hole stopper at each

end. The tube was inclined at an angle of 45°; the lower orifice was connected to a supply of compressed air and the other to a 500 ml. flask by rubber tubing. The compressed air stream set up turbulence within the tube and the continual impacting of the charcoal against the sides of the tube tended to break down aggregates. By varying the air pressure and slope of the tube the size of the dust particles entering the flask could be altered. It was thus found possible to produce in the flask a dust cloud of particles less than 1 μ in diameter. Twenty recently killed bees were exposed to such a cloud for 10 min., the bees being rolled over at intervals to expose fresh areas of cuticle. The treated bees, together with controls, were placed in a desiccator and the rate of water-loss from the two groups compared. In the first 2 hr., the dusted bees lost water at a rate 30 times greater than the controls, and in 24 hr. the average rate was 18 times greater. This greatly enhanced increase in the rate of water-loss using activated charcoal as compared with its effect in Exp. III was considered to be due to a much larger area of the cuticle being brought into contact with the dust. A detailed microscopic examination showed that approximately 40 % of the abdominal and 20 % of the thoracic cuticle appeared to be in actual contact with the dust particles. (These estimates do not include the areas of cuticle covered by the overlapping ridges of the cuticle.) The result of this experiment again seems to rule out abrasion as the main factor in the action of dusts in disrupting the waterproofing mechanism.

Discussion

The results of Exps. I–V clearly show that various dusts can disrupt the cuticular waterproofing mechanism of the worker honey-bee; that the dusts are effective on both living and dead insects, and that they apparently do not need to abrade the cuticle to promote increased water-loss. The importance of considering the surface relief of the cuticle when developing dusting techniques is clearly demonstrated. All the most effective dusts possess powerful adsorbent properties, and when this fact is considered alongside the evidence that the rate of increased water-loss is proportional to the area of cuticle in contact with the dust, it would appear that the dusts act by adsorbing at least one component of the waterproofing layer. This is presumed to be of a lipoid nature.

Alexander *et al.* (1944*b*), when considering the mechanism of dust action, suggested that the epicuticular lipoid film is preferentially attracted to the crystalline forces at the surface of a solid dust particle; it adheres and orients itself on the crystal rather than on the relatively structureless surface of the cuticle, which then ceases to be waterproof. Further work by Wigglesworth (1945) and Beament (1945) (with a variety of insect species which did not include adult hymenoptera showed that this only occurred when the lipoid layer was in the nature of a mobile grease as found in Blattids. When a wax was present, Beament showed that (at the surface of the epicuticle) there was an innermost layer of wax molecules which was specifically oriented and capable of resisting the forces of adsorption exerted by the dusts. Abrasion was held to be necessary to disrupt this orientation; afterwards adsorption might occur on the dust. Hurst (1948), as indicated previously, disagreed with the

main conclusions of Wigglesworth and Beament concerning the mechanism of dust action. Recently, Helvey (1952), using the Mexican Bean beetle, found that dust particles with sharp edges had little or no insecticidal effect whilst others, e.g. Attaclay with no obvious abrasive properties, were highly toxic.

The effect of 'inert' dusts on adult Hymenoptera was studied by Bartlett (1951). He was uncertain whether the dusts acted by abrading the cuticle and suggested the disrupting of the wax film through sorption by dust particles as a possible alternative explanation. Anderson & Tuft (1952) reported that an attapulgite clay was toxic to worker bees kept at a low humidity; this mineral has a high adsorptive capacity and is non-abrasive. It would seem likely, therefore, that in the worker bee and possibly other adult Hymenoptera the waterproofing layer is different from that in the insects examined by Wigglesworth and Beament, and far more susceptible to adsorption by dusts.

PART 2. THE PHYSICAL CHARACTERISTICS OF THE CUTICULAR LIPOID

Experiment VI. The action of chloroform on the epicuticular lipoid of the honey-bee

Wigglesworth (1945) showed that insects placed in an atmosphere of chloroform vapour became less waterproof. He considered that this vapour disorganized the orientation of the wax molecules in the lipoid layer and showed that the effectiveness of the chloroform vapour was directly proportional to the hardness of wax.

The effect of chloroform vapour on the waterproofing mechanism of the worker bee was determined, using the same technique as in previous experiments for measuring the rate of cuticular water-loss. Groups of freshly killed bees were treated as follows: (*a*) group kept at 28° C. in saturated atmosphere of chloroform vapour for 1 hr.; (*b*) treatment as in (*a*) but kept in vapour for 2 hr. Controls were kept at the same temperature. The subsequent increased rates of water-losses over controls were: (*a*) 1·36, (*b*) 1·64.

Further groups of bees were immersed in liquid chloroform at 28° C. for varying periods of up to 1 hr. The bees were gently shaken at intervals, and were then subjected to desiccation after the solvent had evaporated. The increased rates of water loss over controls during the first 2 hr. in the desiccator were for 1 min. exposure, 5·2; 12 min., 11·4; and 1 hr., 37·5. Further tests showed that the extent to which the bees were shaken in the solvent was important; bees kept in chloroform at 28° C. for 20 min. without shaking lost water at a rate 8 times faster than controls; with agitation, the loss was 20 times faster. Treatment in boiling chloroform for 2 min. resulted in a greatly increased rate of water-loss (approximately $\times 60$), and prolonged treatment did not increase this effect.

The results obtained with chloroform vapour indicate that its effect was small. When compared with the results obtained for *Nematus*, *Blatta* and *Rhodnius* by Wigglesworth (1945) it would seem that there is a hard wax present on the epicuticle of bees similar to that on *Rhodnius* and dissimilar to the mobile grease found in *Blatta*. On the basis of his results with *Rhodnius*, Wigglesworth (1945) suggested

that as hot chloroform was required to effect an appreciable wax extraction, and as the wax so obtained was readily soluble in cold chloroform (Beament, 1945), it was possibly protected by another layer termed cement which was only attacked by hot chloroform. In a later publication (Wigglesworth, 1947) the presence of such a layer was confirmed. The present results, whilst following the same trend as those obtained by Wigglesworth with *Rhodnius*, do not appear to suggest that the chloroform ever removes any substance other than wax. The deep ridges or folds on the bee's cuticle will tend to restrict the flowing of the solvent over its surface, particularly at the bottom of the ridge. It has been clearly shown that the effectiveness of the cold chloroform is greatly increased if the insects are shaken in the solvent; such an action would increase the rate of flow of solvent against the cuticle, i.e. its 'washing action'. It is considered, therefore, that the pronounced effect of boiling chloroform is merely due to an extension of this action, the increased temperature having an additional effect on the speed at which the chloroform frees the wax from the underlying protein. When this evidence is considered alongside the fact that the wax layer is readily adsorbed by a dust without mechanical abrasion taking place, it would appear that in the worker bee there is no continuous layer of cement protecting the underlying wax.

Experiment VII. The effect of temperature on permeability

Wigglesworth (1945) using the cuticle of intact insects, and Beament (1945) using films of wax isolated from insect cuticle, showed that the cuticular waxes have fairly definite melting-points, and a 'critical temperature' for the passage of water occurs about 5–10° C. below the melting-point. It was suggested that at this 'critical temperature' the orientation of the wax molecules changes so as to permit the passage of water molecules. The soft waxes showed improved waterproofing after they were heated above their critical temperatures and allowed to cool.

To determine the effect of increases in temperature on the permeability of the bee's cuticle, groups of freshly killed bees were subjected to a range of different temperatures for half hourly periods. The bees were placed in a small tube which was immersed in a thermostatically controlled water-bath. After treatment, each group was placed in a desiccator to cool together with a control group which had been kept at room temperature. The two groups were then weighed and returned to the desiccator and re-weighed after 24 hr. Table 3 shows the average loss in weight for one bee in each group compared with that of the corresponding control group. The difference between these two figures, indicating the effect of the different temperatures, shows that heating the bees up to 58° C. causes a slight increase in waterproofing. Further increases in temperature reverse this effect and above 63·2° C. the decrease in waterproofing becomes very pronounced. Presumably this coincides with the melting of the wax. It would appear then that the critical temperature for the lipoid layer on the epicuticle of the honey-bee is near 59° C., a temperature significantly close to its melting-point, viz. 63–65° C. This further strengthens the hypothesis that the lipoid consists of a hard wax, and its melting-point suggests that it might be similar to beeswax (average m.p. 63° C.).

Table 3. *The effect of temperature on cuticular water-loss*

Temperature ° C.	Average water-loss from one dead bee in 24 hr. in mg. $\times 10^{-1}$		
	Controls	Treated	Difference ±
43·1	24	24	0
55·0	31	24	− 7
57·0	24	21	− 3
58·0	27	23	− 4
59·7	28	36	+ 8
61·8	30	35	+ 5
63·2	43	52	+ 9
65·0	31	51	+20
68·0	34	62	+28

Experiment VIII. The effect of increased temperature on the susceptibility of the lipoid layer to disruption by dusts

A group of dead bees was kept at 57° C. (just below the critical temperature) for 6 hr., the air in the flask containing the bees being kept saturated with water vapour. A similar group was kept at 20° C. for the same period, also in a water-saturated atmosphere. After treatment, half the bees from each group were dusted with Almicide using the technique as in Exps. I–III and the remainder kept as controls. The rates of water-loss of the various groups were determined as in previous experiments. Special care was taken to ensure that both groups were evenly dusted.

Table 4. *The effect of temperature on the action of Almicide*

	Increased rate of water-loss over controls
Bees kept at 57° C.	1·43
Bees kept at 20° C.	3·65

The results in Table 4 indicate that the increased temperature has made the lipoid layer less susceptible to disruption by dust. It is suggested that the increased temperature improved the orientation of the wax molecules in contact with the underlying protein (cf. Beament, 1945).

PART 3. RECOVERY OF IMPERMEABILITY AFTER DUSTING

Wigglesworth (1945) demonstrated that living insects would slowly recover their impermeability after dusting. In *Rhodnius* the degree of recovery depended on whether the dust was removed after application, and it was considered that once the protective wax film had been disrupted by abrasion, recovery could be impeded by the adsorptive action of dust particles on the cuticle.

Experiment IX. Demonstration of recovery after dusting by worker bees

Forty living workers were exposed to a dust cloud of activated charcoal for 1 min. using the technique described in Exp. V; very little dust was visible on the bees. Ten dusted bees were killed and their rate of water-loss compared with controls.

The remainder were caged with a supply of sugar syrup at 30° C. and a relative humidity of 65 %. After 24 hr., ten bees were removed and their rate of water-loss compared with undusted controls which had also been caged, and 48 hr. later the twenty remaining bees were killed and ten of them redusted. The rate of water-loss of each group was then measured and compared with controls.

Table 5. *Variation in water-loss when dusted bees are kept alive for different periods of time*

	Rate of water-loss
Immediately after dusting	4·10
24 hr. after dusting	1·01
72 hr. after dusting	1·03
When redusted	3·10

The results (Table 5) indicate that the dusted bees, when kept alive, completely recovered their impermeability in 24 hr. and that further dusting again produced an increase in water loss. Further tests suggested that bees exposed to the same dust cloud for longer periods did not recover their impermeability, but a high rate of mortality of the test insects occurred and the results were probably not significant.

PART 4. EXPERIMENTS WITH OTHER ADULT HYMENOPTERA

A preliminary investigation was made into the effect of dusts and solvents on insects allied to the honey-bee (other tests not quoted showed no appreciable differences among the three castes of the honey-bee).

Ten workers of three species of *Bombus* and of the wasp (*Vespa germanica*) were killed with hydrogen sulphide and placed in separate flasks. Each group was dusted with Almicide and the excess blown off with a jet of compressed air. The subsequent ratios of water-loss when compared with controls (Table 6) show that the dust had effected an increase in water loss with all species.

Table 6. *Effect of dusting* Bombus *and* Vespa *workers with Almicide*

	Rate of water-loss over controls
Bombus lucorum	1·5
B. hortorum	1·7
B. terrestris	1·4
Vespa germanica	2·9

One group of worker wasps (*V. germanica*) was immersed for 2 min. in boiling chloroform and another in cold chloroform (20° C.) for the same period. Subsequent rates of water-losses were respectively 23·2 and 8·1 times greater than the controls. These figures, though at a different level, are comparable to those obtained with the worker honey-bee.

There seems no reason therefore to suggest that the properties of the epicuticular lipids of the honey-bee are in any way peculiar to that species, but are probably applicable to other Aculeates.

GENERAL DISCUSSION

In the present paper evidence has been obtained which strongly suggests that the waterproofing layer on the cuticle of the worker bee embodies a hard wax probably similar to beeswax. It has been clearly shown that the permeability of the cuticle to water vapour is increased when a variety of dusts are brought into intimate contact with the cuticle, and the evidence suggests that the dusts act by adsorbing wax. The loss of waterproofing brought about by activated charcoal in Exp. V is half that which occurs when the bees are immersed in boiling chloroform (Exp. VI) which should effect the complete extraction of any lipoid material on the surface of the cuticle. As in Exp. V the dust appeared to be in contact with less than 40 % of the total surface of the cuticle; it seems probable, therefore, that the dust and solvent are acting on the same wax layer which cannot be effectively protected by a superficial layer of cement. Beament (1945) found that the thickness of the wax layer on the epicuticle of a range of insect species averaged from 0·2 to 0·3 μ. These figures were obtained by relating the total wax extracted from the surface to the apparent surface area indicated by camera lucida drawings. No account was taken of any folds in the cuticle, and the method could not show whether the wax thickness varied in different regions of the same cuticle. Assuming an average wax molecule to be 100 Å. in length (Muller, 1930) and that all the molecules are oriented vertically, an even wax layer 0·2 μ thick would correspond to at least 20 molecules in depth. The work of Alexander *et al.* (1944*a*, *c*), Wigglesworth (1945) and Beament (1945) clearly shows that it is the innermost compact monolayer of oriented wax molecules which is the main waterproofing barrier.

If in the worker bee the wax layer was approximately 20 molecules thick, then it is difficult to conceive how a dust particle settling on the wax could exert any effect on the innermost layer of molecules. The dust particles would always preferentially attract to their surface the wax molecules not bound and oriented to the underlying protein. It is suggested, therefore, that in the worker honey-bee the wax layer approaches a monolayer in thickness, at least on some areas of the cuticle. If this is the case, we can envisage dust particles settling on the film and when they gravitate to their final resting position, will attract to their surface wax molecules present at the point of contact, thus producing minute gaps in the monolayer. It is probable that the Brown & Escombe 'pinhole' effect (1900) operates in such circumstances, and the production of a large number of such gaps would greatly increase the permeability of the cuticle to water vapour.

In living insects the presence of dust between moving surfaces of the cuticle will tend both to abrade the wax layer and, as Hurst (1948) suggested, bring fresh surfaces of the dust into contact with the wax. It may well be that the main difference between the waterproofing mechanisms in the worker bee and *Rhodnius* is the presence of a continuous cement layer in the latter insect only. The need for abrasion by dusts in *Rhodnius* as demonstrated by Wigglesworth (1945) might then be explained in terms of penetrating the cement layer and not the wax.

SUMMARY

1. Experiments are described which show that the rate of water-loss from living and dead worker bees is increased when a variety of dusts are brought into intimate contact with the surface of the cuticle. The common property of the more effective dusts is their capacity to act as adsorbents. Considerable evidence has been accumulated to suggest that the dusts need not abrade the surface of the cuticle in order to effect an increased water-loss and that the dusts act by adsorbing the epicuticular lipoid.

2. The surface relief of the cuticle of the worker honey-bee is described and the importance of considering this feature of the insect in any experiments dealing with the action of dusts is demonstrated.

3. An evaluation of the physical properties of the epicuticular lipoid has indicated that it contains, or possibly entirely consists of, a hard wax similar to beeswax.

4. The action of the dusts and of chloroform suggests the absence of a continuous cement layer, and it is suggested that the wax approaches a monolayer in thickness, at least on some areas of the cuticle.

5. Living worker bees were shown to be capable of recovering their impermeability after dusting.

6. The type of waterproofing mechanism described in the honey-bee is not thought to be peculiar to that species. It is probably present in other Aculeates.

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OBSERVATIONS ON THE RESPIRATORY PHYSIOLOGY AND ON THE HAEMOGLOBIN OF THE POLYCHAETE GENUS *NEPHTHYS*, WITH SPECIAL REFERENCE TO *N. HOMBERGII* (AUD. ET M.-EDW.)

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INTRODUCTION

Of the polychaete family Nephthydidae, comprising the single genus *Nephthys*, several species have invaded the intertidal zone (penetrating to about M.S.L.) and may, as burrowers, be locally abundant on sandy shores. The present paper gives the results of a preliminary investigation of the respiratory arrangements in this genus.

Barcroft & Barcroft (1924) reported on the positions of the spectral absorption bands of haemoglobin from *Nephthys* sp., but remarked that haemoglobin solution was much more readily obtainable from *Arenicola*, a fact which may account for the subsequent neglect of *Nephthys* by workers on invertebrate respiratory pigments. Nevertheless numbers adequate for experimental purposes are readily obtainable and the species mentioned in this paper are easy to keep in aquaria with sand in which they may burrow.

In view of the small amount of published information on this genus, some original observations on the mode of life of *Nephthys* have been made, and a summary of the relevant aspects is given below. These facts are essential to the interpretation of the experimental data.

Nephthys is regarded as an errant polychaete. It has well-developed swimming powers but also a pronounced burrowing habit with which is associated no special morphological adaptation except for the enlarged pharynx which is used to great effect in the process of burrowing. When placed in a sand-floored aquarium it immediately burrows and comes to rest eventually with its anterior end at or a few centimetres below the sand surface. Upward excursions are made when necessary to open up the burrow to the overlying water. I have never seen worms under these conditions leave the sand spontaneously and swim freely in the water.

The walls of the burrow are not at all consolidated with mucus and the whole is very impermanent, the animals moving about from time to time in the sand. They seldom penetrate more than 15 cm. below the surface in the natural habitat.

In all the species studied parts of the body surface are ciliated in the manner described by Coonfield (1931), so as to produce antero-posterior water currents flowing in the space enclosed by the neuro- and notopodia and the lateral wall of the burrow. The segmental gills hang down into this space and there can be little doubt that the current which has been observed in sand-floored aquaria, is of

respiratory significance. For this reason the absence of any mucous lining to the burrows appears to determine the close proximity of the worms to the sand surface (cf. *Nereis diversicolor* and *Nerine cirratulus* whose burrows are substantially lined with mucus and which are commonly found at considerably greater depths than *Nephthys*). The lack of mucous lining also determines the collapse of the mouth of the burrow when the tide ebbs and the isolation of the worm from any oxygen supply outside the sand.

It is these features of the normal habitat which lend interest to the estimation of interstitial oxygen in the sand and to the study of the properties and distribution of the respiratory pigment.

As the unspecialized nature of the adaptation of *Nephthys* spp. to littoral life has become apparent, it has seemed desirable to attempt a comparison of the respiratory arrangements in this genus with those of *Arenicola*. Original observations of the oxygen content of the water remaining in exposed *Arenicola* burrows are given, and the comparison between the genera has been developed in the discussion.

MATERIALS AND METHODS

The animals were collected from three localities on the Yorkshire coast, namely 'The Landing' at Robin Hood's Bay, the north end of Filey Bay and the old harbour at Scarborough. In these places the species found are *Nephthys hombergii*, *N. caeca*, *N. cirrosa* and *N. longosetosa*, identified according to the descriptions of Fauvel (1923). In all the above places *N. hombergii* is the most abundant and has been most closely studied.

Various quantitative techniques have been used. A simple method has been devised for obtaining samples of the interstitial water from the sand, which completely avoids contact between the sample and the atmosphere. Oxygen concentrations of these samples were determined by the micro-Winkler technique of Fox & Wingfield (1938) after pre-treatment of the samples according to Alsterberg's method (1926). The positions of the absorption bands of the haemoglobin spectra were determined by the use of the Hartridge reversion spectroscope, while haemoglobin concentration was determined spectrophotometrically on the pyridine haemochromogen derivative. The method of obtaining the oxygen dissociation curve data was based on those of Hill (1936) and of Allen, Guthe & Wyman (1950). Details of the application of these methods to the present problems are given in the appropriate sections.

OXYGEN CONTENT OF THE INTERSTITIAL WATER

Method

Since *Nephthys* is sealed in the sand when the latter is exposed by the tide, it is obviously very desirable to have a reliable estimate of the oxygen content of the interstitial water. An attempt to measure this factor was made by Borden (1931) in connexion with her work on the respiratory physiology of *Arenicola*. She described a sampling method which involved allowing the interstitial water to drain into a

tube pushed into the sand, and subsequently making an allowance for contamination with the atmosphere after the determination of a control. No steps were taken to deal with reducing substances in the water other than hydrogen sulphide, or to allow for oxygen in the reagents, and her failure to detect oxygen in excess of that likely to have been introduced by contact with the air cannot therefore be regarded as proving that the water was completely oxygen-free. A simple and more direct approach to the sampling problem seemed desirable.

In the present work the samples were drawn into 20 ml. all-glass syringes through a special cannula attached to a standard Luer hypodermic needle mount. The cannula was a 20 cm. length of 15 s.w.g. stainless steel tubing, and attached just below and projecting a few millimetres beyond the tip was a small pointed cap, in shape somewhat like a miniature candle snuffer. This protected the tip of the cannula while it was being pushed into the sand and also carried down immediately in advance of the tip a small pellet of cotton-wool. In this way the cannula could be inserted into the sand without becoming choked and the syringe could be filled with water by gently withdrawing the plunger. At first a small quantity of water was drawn in to displace the air-bubble in the dead space. Next the cannula was re-inserted into the sand to the required depth and the full sample of 10 or 20 ml. taken. The cannula was then removed and the syringe sealed without air bubbles by means of a short piece of rubber tubing and a piece of glass rod.

The first samples taken by this method and analysed by the technique of Fox & Wingfield (1938) gave titres significantly below the value for the blanks (as determined by Krogh's method (1935)), representing the oxygen dissolved in the reagents. From this it was concluded that the sample contained reducing substances which interfered with the reagents, and accordingly Alsterberg's (1926) modification of the Winkler method was adopted. The pre-treatment with Alsterberg's reagents was carried out in the sampling syringe, and a smaller (1·5 ml.) sample was subsequently withdrawn into the syringe-pipette for the normal Fox & Wingfield procedure.

The same techniques have been used to obtain and analyse samples of the water which remains in the burrows of *A. marina* after the sand is exposed by the tide, using polythene instead of steel tubing for the sampling-syringe cannula. Inserted into the tail shaft the polythene cannula readily followed the natural configuration of the burrow, and a 10 ml. water sample was easily withdrawn.

Results

Interstitial water samples from depths of 7·5 and 15 cm. were obtained at Filey, Scarborough and Robin Hood's Bay, in the latter instance at various intervals after the exposure of the sand by the tide. The results of the determination of oxygen content of these samples (Table 1) in each case indicate the presence of a definite though small concentration of oxygen, the values ranging from 0·11 to 0·35 ml. of oxygen per litre. No steady drift of oxygen tension with exposure time is evident; neither is there a significant difference between the samples from depths of 7·5 and 15 cm.

Table 1. *Concentration of dissolved oxygen in the interstitial water of the sand*

Stations: A, 'The Landing', Robin Hood's Bay; B, Filey sands, close to the Brig; C, The Old Harbour, Scarborough. All about M.S.L.)

Station	Hours after exposure	Depth (cm.)	Dissolved oxygen (ml./l.)	Station	Hours after exposure	Depth (cm.)	Dissolved oxygen (ml./l.)
A	$-\frac{1}{4}$	7·5	0·31	B	4	7·5	0·27
	$-\frac{1}{4}$	15·0	0·27		4	7·5	0·33
	0	12·5	0·20		4	15·0	0·22
	0	12·5	0·13		4	15·0	0·24
	0	7·5	0·22				
	0	15·0	0·35				
	3	12·5	0·22	C	4	7·5	0·35
	5	7·5	0·26		4	7·5	0·27
	5	15·0	0·20		4	15·0	0·24
	6	12·5	0·26		4	15·0	0·33
	6	7·5	0·15				
	6	15·0	0·11				

Mean of all samples = 0·25 ml./l., corresponding to 6·7 mm. tension of oxygen at 15° C.

Table 2. *Concentration of dissolved oxygen in the residual water of* Arenicola *burrows*

(All samples from burrows on 'The Landing', Robin Hood's Bay at about M.S.L.)

Hours after exposure	Dissolved oxygen (ml./l.)	Hours after exposure	Dissolved oxygen (ml./l.)
0	1·48	2	0·43
0	0·76		
		$3\frac{1}{2}$	0·67
$\frac{1}{4}$	0·85	$3\frac{1}{2}$	0·49
$\frac{1}{4}$	1·03		
		5	0·43
2	0·45	5	0·56

Mean of the two 5 hr. samples = 0·50 ml./l., corresponding to 13·4 mm. tension of oxygen at 15° C.

The oxygen dissolved in the reagents, which is allowed for by deducting the value of a blank titration, accounts for more than half of the oxygen determined in every case. However, since replicate blank determinations agree within ±4%, the variation in the results is almost certainly real and representative of slight local fluctuations in the density of interstitial micro-organisms. When the tide ebbs *Nephthys* is therefore sealed in a medium in which the oxygen concentration corresponds to a partial pressure of less than 10 mm. of Hg. The mean value of all the determinations corresponds to a partial pressure of 6·7 mm at 15° C. It is interesting to note that the values for the Scarborough samples, where conditions appear to be 'fouler', fall within the range of values for samples from the cleaner sands at Robin Hood's Bay and Filey.*

* The substrate in Scarborough harbour contains much higher proportions of silt and organic matter than those at Robin Hood's Bay or Filey and the water is polluted by fishery activities. The *Nephthys* population consists entirely of *N. hombergii*, whose tolerance of silt has been noted by various authors (e.g. Southward, 1953). The population is also remarkable for a high concentration of coelomic haemoglobin; the absence of other *Nephthys* spp. may be connected with respiratory problems.

The results of oxygen determinations on water samples from exposed *Arenicola* burrows on the 'Landing' at Robin Hood's Bay are shown in Table 2. In spite of the limited number of samples it is clear that even after 5 hr. exposure the water in the burrow may contain twice as much oxygen as the interstitial water in the adjacent sand (cf. Tables 1 and 2).

The partial pressure of oxygen corresponding to the mean of the two 5 hr. samples is 13·4 mm. at 15° C. The two samples taken from separate burrows (as were all the duplicates) at zero time indicate already a sharp fall from full saturation (5·8 ml./l. at 15° C.) and there is a large difference between them. This presumably reflects the respective amounts of activity by the two worms since the occasion on which each performed its last previous bout of respiratory irrigation.

THE RESPIRATORY PIGMENT

Nephthys has a closed blood vascular system containing a red pigment but possesses also a red pigment in the coelomic fluid. Attention was drawn to the latter by the conspicuous pink colour of the everted pharynx of *N. hombergii* from Scarborough. This was due to the inflow into the extrovert during protrusion, of a coelomic fluid which in these worms contained a relatively high concentration of pigment. The presence of the pigment in the coelomic fluid was later confirmed in worms from Robin Hood's Bay and Filey and in all the four species.

Practically all the coelomic fluid can easily be removed by inserting a long fine pipette in the region of the 20th segment between the body wall and the dorsal side of the gut. This can be done without visible injury to the blood vessels. By centrifuging the extracted fluid a perfectly clear pink solution can be obtained, the deposited debris consisting largely of gametes.

The collection of blood is more tedious because of the small size of the blood vessels. The major part of the coelomic fluid having been removed, the worm is pinned out on a dry wax block and the body cavity opened dorsally from head to tail. The remaining coelomic fluid is removed with filter-paper. The dorsal blood vessel and the longitudinal vessels serving the extrovert are then punctured, taking great care not to damage the gut. The animal is set on one side for 10 min. or so, during which time the blood gently oozes into the body cavity from which it can be collected with a fine pipette. This technique yields but a fraction of the total blood, in one case 0·35 ml. from thirty-two *N. hombergii* aggregating 49·3 g. The purity of blood samples obtained in this manner may be questioned, but provided the body cavity is adequately dried with filter-paper after opening the worm and the gut wall is not perforated, the degree of contamination with other fluids is very small and any solid material collected can be removed by centrifuging.

Microscopic examination of fresh blood and coelomic fluid and of smear preparations failed to show any pigmented corpuscles, although there was often an abundance of non-pigmented cells in the coelomic fluid. There was no separation of 'plasma' and pigment on standing nor could the pigment in either case be concentrated by centrifuging the fresh fluid at 5000 r.p.m. It was therefore concluded that it is in free solution in both the coelomic fluid and the blood.

Both the blood and the coelomic fluid from *N. hombergii* and *N. caeca* change to a more bluish hue on deoxygenation. In each case the colour reverts to the original on shaking with air. Spectroscopic examination of both fluids shows: (*a*) a pair of fairly sharp absorption bands in the yellow-green region in the oxygenated state; (*b*) a single diffuse band of intermediate position in the de-oxygenated state. Both pigments are therefore characterized as haemoglobins. The positions of the absorption bands of the oxyhaemoglobins of both blood and coelomic fluid from *N. hombergii* and *N. caeca* have been determined with the Hartridge reversion spectroscope. In each case the readings were compared with alternate readings on sheep haemoglobin of similar dilution. The mean wave-length values obtained are set out in Table 3.

Table 3. *Positions of spectral absorption bands of* Nephthys *oxyhaemoglobins*

(Determined with the Hartridge reversion spectroscope —Å. units)

Species	Vascular		Coelomic	
	α	β	α	β
N. hombergii	5751±3	5387±5	5750±3	5389±8
N. caeca	5755±3	5391±7	*5757±5	5396±6
Sheep	5764±3	5402±3		

* This figure agrees with that given by Barcroft & Barcroft (1924) for coelomic haemoglobin of *Nephthys* sp.

It is apparent that the α-bands of both the vascular and coelomic haemoglobins are shifted towards the blue end compared with sheep haemoglobin, and thus resemble the α-band of *Arenicola* oxyhaemoglobin (5746 Å., Barcroft & Barcroft, 1924), although the shift in the case of *Nephthys* haemoglobin is not so great. The differences between vascular and coelomic haemoglobins in each species of *Nephthys* and the differences between the species are not significant.

A number of spectrophotometric determinations of coelomic haemoglobin have been made using the pyridine haemochromogen derivative. Coelomic fluid from a number of worms was pooled until a large enough sample was obtained to make a single determination. Between six and twelve worms were needed to give 0·5–1·0 ml. of coelomic fluid after centrifuging. The volume of clear solution having been carefully measured it was diluted to exactly 5 ml. with distilled water and poured into a centrifuge tube. To this 0·5 ml. of normal sodium hydroxide was added, and the mixture was then heated in a water-bath for 3 or 4 min., to denature the globin and oxidize the haem. After a thorough shaking, 1 ml. of pyridine was added and the contents of the tube shaken again. The solution was then centrifuged until a perfectly clear supernatant fluid could be decanted. A trace of pure sodium hydrosulphite was finally added to reduce the haematin and bring about the formation of the pyridine haemochromogen. The second centrifuging was necessary because the sodium hydroxide brought down a flocculent white precipitate. Since some of the haematin might be adsorbed on to this precipitate, the clear haemochromogen solution was decanted and the precipitate resuspended in distilled water

with a little sodium hydroxide, pyridine and a trace of hydrosulphite. When this suspension was examined with a hand spectroscope no absorption bands were seen, so it was concluded that all the haem from the original sample of coelomic fluid was present in the decanted supernatant fluid in the form of pyridine haemochromogen.

A figure representing the concentration of pyridine haemochromogen and hence the concentration of haemoglobin was obtained by measuring the light absorption of the solution in a 1 cm. cell in the Hilger Biochem Absorptiometer. The absorption was measured over the wave-length range transmitted by Chance glass filters OG 1 and OY 2 which give a maximum transmission in the region of 550–555 mμ, corresponding to the principal absorption band of the haemochromogen. Absolute values for haemoglobin concentration expressed in terms of oxygen capacity (μl. per ml.) were obtained by reference to a calibration curve. This curve was made by plotting the measured light absorption of haemochromogens prepared from known dilutions of sheep haemoglobin whose oxygen capacity had been determined with the Van Slyke apparatus. Knowing the degree of dilution of the original coelomic fluid in preparing the haemochromogen, the original oxygen capacity of the coelomic fluid could be calculated. From the results of these determinations, (Table 4), it appears that the concentration of coelomic haemoglobin in the Scarborough population is some $2\frac{1}{2}$ times that of the pigment in the Robin Hood's Bay worms. There is a considerable variation between specimens in each population, which is unfortunately masked to some extent by the need to pool the coelomic fluid from a number of worms in order to get a determination.

Table 4. *Oxygen capacity of coelomic haemoglobin solution from* Nephthys hombergii

(Pooled samples from six to twelve worms)

Locality	O_2 capacity (μl./ml. of fresh coelomic fluid)					Mean
'The Landing' R.H.B. Scarborough harbour	1·66 —	1·60 3·40	1·26 4·98	2·78 6·16	2·06 —	1·88 4·85

Table 5. *Oxygen-combining potential of coelomic fluid per gram (wet weight) of worm*—Nephthys hombergii

Locality	Wt. of worm (g.)	Vol. coel. fl. (ml.)	Total O_2 (μl.)	O_2 (μl.) per g.
R.H.B. Scarborough	21·6 27·5	3·3 4·0	6·6 17·9	0·31 0·65

In a number of cases worms were weighed (after drying on filter-paper) before the coelomic fluid was removed. It is thus possible to make a few rough calculations of the oxygen-combining potential of the coelomic fluid per g. of worm. These data grouped according to the locality from which the worms were taken, are shown in Table 5.

An attempt has also been made to estimate the total haemoglobin content of *N. hombergii* from Robin Hood's Bay. Worms were cut into small pieces with scissors and ground in a mortar with clean sand and a little sea water. The total contents of the mortar were then transferred to a tube and centrifuged at about 5000 r.p.m. for 20 min. The supernatant fluid was not quite clear, but it was decanted and treated with sodium hydroxide and pyridine. In three cases it was possible to get perfectly clear solutions after the final centrifuging, and in each of these cases a little haemochromogen could be detected with the hand spectroscope on resuspending the solid material from the bottom of the centrifuge tube. However, when a final clear solution was obtained it was decanted, its volume measured, some hydrosulphite added and the light absorption measured. Having read off the oxygen capacity of the extract from the calibration curve and knowing its total volume, the total oxygen-combining potential of the whole extract could be calculated. In Table 6 this is related to the wet weight of the worms from which the extract was made. These figures are minimal estimates of total haemoglobin since some of the pigment is lost in each of the two centrifuging processes. However, it is unlikely that the true values are more than 50% higher.

Table 6. *Oxygen-combining potential of whole worm extracts of* Nephthys hombergii—*all from Robin Hood's Bay*

Wt. of worm (g.)	Vol. extract (ml.)	O_2 capacity (μl./ml.)	Total O_2 (μl.)	O_2 (μl.) per g. of worm
2·7	5·5	2·04	11·2	4·1
2·9	11·6	0·76	8·8	3·0
1·7	5·6	0·78	4·4	2·6

The oxygen consumptions of a number of *N. hombergii* have been determined at 15° C. by means of a multiple dropping-mercury electrode. (The design and use of this instrument will form the subject of a separate paper.) Worms were enclosed singly in small glass bottles containing sea water and allowed to deplete the dissolved oxygen. The oxygen concentration in the bottles was determined at half-hourly intervals over a period of 4–5 hr. It was then possible to calculate the oxygen consumption of individual worms at a number of different oxygen concentrations. As the oxygen concentration of the medium fell from 4·5 to 1·25 ml./l. the mean oxygen consumption fell from 80 μl./g. (wet weight)/hr. to 20 μl./g./hr.

From the various groups of data presented above it is now possible to make a very rough estimate of the value of the combined vascular and coelomic haemoglobins in providing a potential oxygen-store to meet the needs of the worm at times of oxygen lack. Assuming the lower level of metabolism quoted above, it will be seen that the overall oxygen-combining potential (Table 6) of the order of 2·5–4·0 μl. of oxygen per g. of worm will not meet the needs of the animal for more than 7–12 min. The contribution of the coelomic haemoglobin in the case of the Robin Hood's Bay worms will suffice for about 1 min., while in the case of the Scarborough worms this figure may be increased to about 2 min. (see Table 5).

Whatever role haemoglobin may play in the life of *N. hombergii* it is quite clear

from the above considerations alone that it cannot usefully act as an oxygen store in the sense that it can materially help the animal over the period of exposure by the tide. Neither can the superior coelomic haemoglobin concentration in the Scarborough population be concerned with providing additional oxygen storage capacity.

THE OXYGEN DISSOCIATION CURVES OF *NEPHTHYS* HAEMOGLOBINS

Method

The data for the construction of the oxygen dissociation curves of coelomic and vascular haemoglobin from *N. hombergii*, were obtained by the use of Hill's method (1936). In the case of the vascular haemoglobin, which was available in much smaller quantities of an initially more concentrated solution, Hill's tonometer was replaced by one of the form described by Allen *et al.* (1950), and the method of adding oxygen to the system was changed accordingly.

Freshly collected coelomic fluid and blood, usually obtained from the same batch of worms, were diluted fourfold with M/20 phosphate buffer mixture to give pH 7·4, and then centrifuged. The perfectly clear haemoglobin solutions were decanted and either used immediately or stored in the deoxygenated state in contact with pure nitrogen at 4° C. No solutions older than 4 days were used, and at this age repeat determinations gave the same results as did the initial ones. Two samples, one of coelomic and one of vascular haemoglobin, were rebuffered after a set of measurements at pH 7·4, by adding extra potassium dihydrogen phosphate to give pH 7·0. All determinations were carried out at 15° C.

Because of the limited amounts of *Nephthys* pigment available sheep haemoglobin solution was used in the comparison cups of Hill's apparatus. The absorption spectra of sheep and *Nephthys* haemoglobin solutions of equal concentration are sufficiently alike for matching to be carried out satisfactorily. The oxygen capacity of the sheep haemoglobin solutions was determined with the Van Slyke apparatus and provided a standard from which the oxygen capacities of the *Nephthys* samples could be derived. Hill's tonometer was modified slightly by extending the bottom of the Thunberg tube so that it had the form of an inverted T; this increased the thickness of the solution and made it easier to work with the rather dilute coelomic haemoglobin solutions.

Results

The percentage saturations and the calculated equilibrium oxygen tensions for both coelomic and vascular haemoglobin samples are set out in Table 7. The dissociation curves constructed from these data are shown in Fig. 1.

The first point of interest about the dissociation curves is the absence of a point of inflexion which characterizes the majority of dissociation curves which have been determined. If each set of data is plotted in the form log HbO_2/Hb against log pO_2 the result should be a straight line whose slope represents the value of 'n' in Hill's equation. The data for the coelomic samples give, on this basis, a very close approximation to a straight line of slope = 1; i.e. the coelomic dissociation curves appear to be rectangular hyperbolae (the form of curve given by Hill's equation when 'n' = 1). The data for the vascular haemoglobin samples give a pair of curves

which depart from slope = 1 at the higher oxygen tensions and in fact give a much better fit to a line representing 'n' = 1·2.

Table 7. *The dissociation of* Nephthys *oxyhaemoglobins*

(Percentage saturations and corresponding equilibrium oxygen tensions (in mm. of Hg) of samples of vascular and coelomic haemoglobin from *Nephthys hombergii* at pH 7·4 and 7·0 and at 15° C.)

Pigment	pH	Dissociation equilibrium data							
Coelomic	7·4	% sat.	18·0	27·5	33·5	45·0	52·5	62·5	78·5	90·0
		pO_2	1·84	3·25	4·62	6·12	8·45	11·9	17·5	29·8
		% sat.	16·5	25·0	42·5	62·0	77·0			
		pO_2	1·90	3·40	5·46	9·88	23·0			
		% sat.	12·5	20·0	30·5	40·0	50·0			
		pO_2	0·84	1·96	3·46	5·65	11·8			
		% sat.	15·0	27·0	40·0	60·0	75·0			
		pO_2	1·15	2·82	5·54	8·90	15·8			
	7·0	% sat.	17·5	30·5	42·5	54·0	71·5	86·0		
		pO_2	1·11	2·26	3·85	6·31	10·5	19·7		
Vascular	7·4	% sat.	30·0	38·0	52·5	59·5	66·0			
		pO_2	2·80	3·97	5·12	6·30	8·63			
		% sat.	17·5	25·0	37·5	44·0	50·0	58·5		
		pO_2	1·17	2·35	3·53	4·71	5·89	7·06		
		% sat.	25·0	47·5	60·0	70·0	80·0			
		pO_2	2·31	4·62	6·94	9·26	11·6			
	7·0	% sat.	12·5	22·5	40·0	55·5	65·0	75·0		
		pO_2	1·18	2·37	4·74	7·11	9·48	11·9		

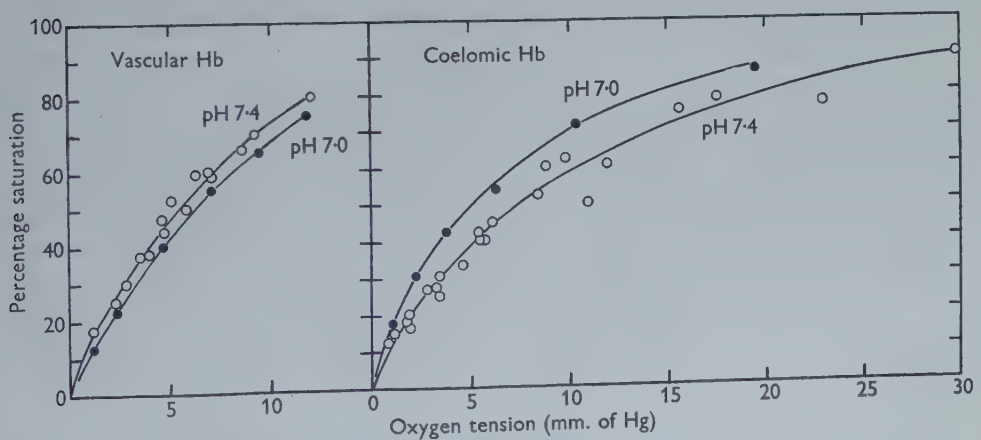

Fig. 1. The oxygen dissociation curves of the vascular and coelomic haemoglobins of *Nephthys hombergii* at pH 7·4 and 7·0 and at 15° C.

It is possible that both the shapes of the dissociation curves and the oxygen affinities of the pigments (relative positions of the curves along the abscissa) may have been affected by diluting the original solutions. Hill & Wolvekamp (1936), who studied the dissociation equilibria of a number of mammalian haemoglobin solutions, found that at dilutions of 1 in 200 all the curves were markedly shifted to the left (oxygen affinity increased) compared with whole blood. In these cases

they were studying in solution pigments whose normal sites are intracellular; the displacement of the curves of dilute suspensions of intact corpuscles was less marked. On the other hand, comparison of the dissociation curves of *Arenicola* haemoglobin obtained by Barcroft & Barcroft (1924), using very dilute blood, and by Wolvekamp & Vreede (1941), using whole blood, clearly indicates that neither the shape of the curve nor the oxygen affinity of this pigment is influenced by dilution. It therefore seems unlikely that in the present examples the moderate dilution (1 in 4) would have appreciably altered the positions of the curves or their shapes. However, it should be borne in mind that the true dissociation curves of *Nephthys* coelomic and vascular haemoglobins may be slightly to the right of those shown.

Although both vascular and coelomic haemoglobin dissociation curves at pH 7·0 are based on a small number of points, there appears in each case to be a small but significant Bohr effect. It is remarkable, however, that in the case of the vascular pigment the effect of increased acidity is to shift the curve to the right in the usual manner, while for the coelomic pigment the effect is reversed. Reversed Bohr effects are very unusual among haemoglobins at least above pH 6·5; the blood of the tadpoles of *Rana catesbiana* (McCutcheon, 1936) and the perenteric haemoglobin of *Ascaris lumbricoides* (Davenport, 1949) are the only examples I have come across in the literature. As a result of this curious situation the oxygen affinities of the two *Nephthys* pigments are comparable at pH 7·0, but at pH 7·4 the vascular pigment has a substantially higher oxygen affinity than the coelomic haemoglobin, the oxygen tensions for 50% saturation being 5·5 and 7·5 mm. respectively.

DISCUSSION

It appears from my observations on the mode of life of *Nephthys* that at least in the littoral species here considered, the greater part of the animal's life is spent below the surface of the sand. The burrow which is of a very impermanent nature can be irrigated with well-aerated sea water while the sand is covered by the sea. But when the sand is exposed to the air the mouth of the burrow collapses and the worm is sealed in. The dissolved oxygen in the interstitial water of the sand corresponds to a tension of about 7 mm. of Hg throughout the period of exposure. It is clear that intertidal species of *Nephthys* are regularly exposed to conditions of poor oxygen supply, and that the respiratory arrangements may be of considerable interest.

The four species of *Nephthys* which have been examined have haemoglobin in free solution in both the blood and the coelomic fluid.* It might be suggested that the freely dissolved haemoglobin in the coelom of *Nephthys* has simply leaked out of the vascular system. It should be emphasized, however, that its occurrence here is a constant feature of the populations examined on the Yorkshire coast. Further, the coelomic haemoglobin differs from the vascular pigment in having (*a*) a reversed

* The existence of a respiratory pigment in both the blood and the coelomic fluid is rare (Romieu, 1923, mentions only *Terebella lapidaria* and *Travisia forbesii*), but an extracellular coelomic pigment appears to be unique. This is neatly correlated with the presence in *Nephthys* of protonephridia and blind coelomostomes (Goodrich, 1945).

Bohr effect, and (*b*) at pH 7·4, which probably represents the *in vivo* condition, a substantially lower oxygen affinity. The oxygen affinity difference is not likely to be due to the difference in haemoglobin concentration between the blood and the coelomic fluid, because in haemoglobins which do show dilution effects it is the stronger solutions which have the lower oxygen affinities (Hill & Wolvekamp, 1936). Thus the evidence at present available indicates that the coelomic and vascular haemoglobin molecules of *Nephthys* are different.

At present no suggestion can be made regarding the possible significance of the difference between the oxygen affinities of the two pigments. In so far as they have any contact with each other there will be a tendency for oxygen to be transferred from the coelomic fluid to the blood if they are at the same oxygen tension. The relationships between vertebrate haemoglobin and myoglobin and between the haemoglobins of foetal and maternal blood immediately spring to mind, but it would be idle to speculate about the present difference in oxygen affinity and in the Bohr effect, in the absence of detailed knowledge of the anatomy of the vascular system in *Nephthys*. Neither is it possible, at present, to make any suggestion regarding the difference in concentration of haemoglobin in the coelomic fluids of the Scarborough and Robin Hood's Bay populations of *N. hombergii*. Studies of the respective environmental conditions are being pursued.

About the physiological significance of the general shape and position of the *Nephthys* dissociation curve some tentative suggestions can be offered. In Fig. 2 are shown the dissociation curves of the vascular and coelomic haemoglobins of *Nephthys* at pH 7·4 and 15° C. and the dissociation curve of the haemoglobin of *Arenicola marina* at pH 7·5 and 19° C. (from Wolvekamp & Vreede, 1941). At 15° C. the *Arenicola* curve would lie a little further to the left. Also indicated in Fig. 2 (by short vertical lines above the abscissa) are the levels of oxygen tension which have been found in the interstitial water of the sand and in the residual water of *Arenicola* burrows respectively (calculated for 15° C.). Let it be assumed that there will be an oxygen gradient of about 10 mm. pressure between the external medium and the arterial blood. (Fox (1945) found gradients of this order in *Chironomus* larvae and in *Tubifex* at limiting tensions of oxygen uptake, though in a form like *Arenicola* with well-developed gills the gradient may be rather less.) Then it is clear that *Arenicola* blood can be almost fully saturated with oxygen from the residual water in the burrow even after 5 hr. exposure. On the other hand, the only oxygen available to *Nephthys* during the exposure period (that in the interstitial water) is at such a low tension as to be virtually unusable. In these conditions the very steep dissociation curve and high oxygen affinity of the *Arenicola* haemoglobin may be seen as an adaptation which enables the animal to survive the exposure period with the minimum of anaerobic metabolism. Whereas in *Nephthys*, metabolism during the exposure period will of necessity be predominantly anaerobic and there will be no selection pressure leading to the evolution of a pigment with a very steep dissociation curve.

There are other animals which are from time to time exposed to conditions of complete or almost complete oxygen lack and which yet have pigments of very high

oxygen affinity. One example is to be found in larvae of the *Chironomus plumosus* group. Estimates of the oxygen tension for half-saturation in this species are 0·2 mm. (Leitch, 1916) and 0·6 mm. (Fox, 1945), while the pigment remains partly oxygenated *in vivo* down to 13 mm. external oxygen tension (Fox, 1945). Ewer (1942) recorded dissolved oxygen concentrations down to 0·3 ml./litre in ponds in which numerous chironomids were to be found; this corresponds to an oxygen tension of about 7 mm. at 20° C. and the haemoglobin of these animals must have been permanently deoxygenated so long as the oxygen tension in the water remained at this low level. Walshe (1950) has shown that the function of haemoglobin in

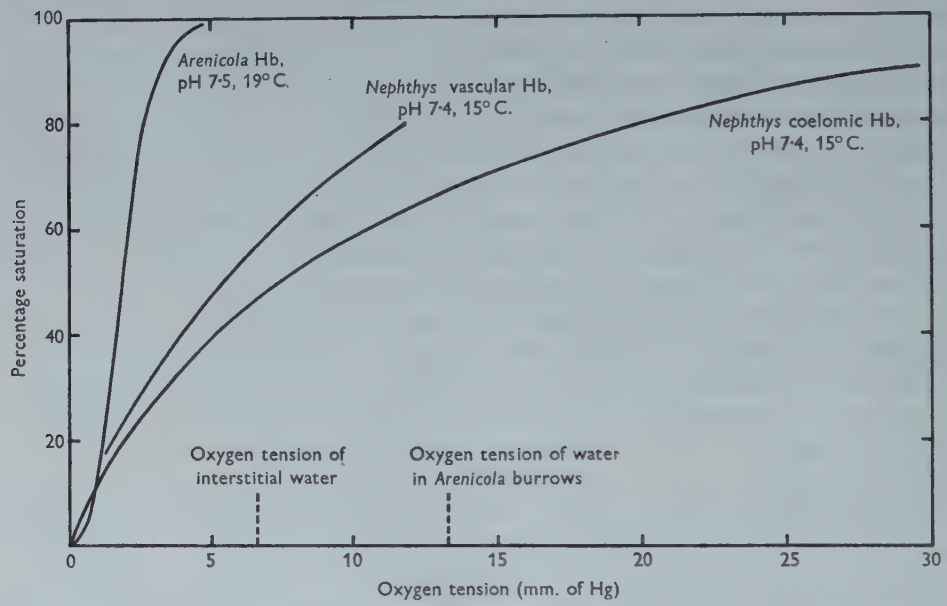

Fig. 2. Comparison of oxygen dissociation curves of the vascular and coelomic haemoglobins of *N. hombergii* at pH 7·4 and 15° C. and of the haemoglobin of *Arenicola marina* at pH 7·5 and 19° C. (*Arenicola* curve from Wolvekamp & Vreede, 1941). The levels of interstitial oxygen tension and of oxygen tension in the residual water of exposed *Arenicola* burrows are indicated by vertical lines above the abscissa.

Chironomus plumosus is to facilitate (*a*) feeding and respiratory irrigation of the burrow during periods of gradual oxygen depletion, and (*b*) recovery, from completely anaerobic metabolism, under conditions of partial oxygen-lack (above 7 % air saturation, i.e. about 11 mm. oxygen tension). Thus for an animal exposed to gradual changes from oxygen sufficiency to complete oxygen lack and vice versa, there are periods (often very extended ones in fresh-water habitats) when oxygen transport at low tensions is both possible and desirable. A respiratory pigment with a steep dissociation curve and high oxygen affinity is then obviously advantageous. On the sea shore an unspecialized burrower like *Nephthys* never has the opportunity for oxygen transport at low tensions because the tension of the available oxygen changes too rapidly from an adequate to a functionally unusable level and vice versa.

Arenicola, on the other hand, with its specialized mode of life can probably obtain oxygen throughout the exposure period by virtue of the conditions in its consolidated burrow and of the adaptation of its respiratory pigment.

The idea of a storage function, originally postulated for *Arenicola* haemoglobin by Barcroft & Barcroft (1924), has been criticized by a number of workers in more recent years (see especially Wolvekamp & Vreede, 1941) on the grounds of the inadequacy of a store calculated to last about an hour. This is a very pressing argument when it is realized that *Arenicola* is often to be found almost as high on the shore as H.W.N.T. (Brady, 1943; Watkin, 1942), and that in this position it will be exposed for over 9 hr. Hecht (quoted by van Dam, 1938) found *Arenicola* in places which were only covered by spring tides. The possibility envisaged in the above speculations, of oxygen transport at low tensions during the period of exposure, offers a satisfactory alternative to the storage hypothesis. The attribution of a storage function to the haemoglobin of *Nephthys* is ruled out by the calculation (see above) that the total combined oxygen in this case would meet the animal's needs for a period of the order of only 10 min.

This speculation on the difference in respiratory arrangements between *Nephthys* and *Arenicola* suggests a number of directions in which further experimental work is needed to test the hypothesis. The use of the CO-method, notably developed by Prof. H. Munro Fox and his school, should confirm (or otherwise) the idea of potential low or high tension oxygen transport systems* in *Arenicola* and *Nephthys* respectively. It is desirable to have determinations of actual oxygen gradients across the body wall in each case under various external oxygen conditions. The need for a low tension oxygen transport system must be further examined in the light of the observations by Wells (1949) of intermittent aerial respiration by *Arenicola* in glass U-tubes containing 'stagnant' water. It would also be most interesting to have an estimate of the oxygen debt incurred in individuals of both species after a given period of exposure in the natural habitat. Experiments are being planned to throw light on these points. If the hypothesis is borne out by further experiments it would appear that despite the similarity of habitat, *Nephthys* and *Arenicola* represent widely different respiratory types, the former unspecialized and the latter highly specialized in its adaptation to littoral life.

SUMMARY

1. Littoral representatives of the genus *Nephthys* are described as burrowing forms, able to irrigate their burrows with well-aerated sea water except when the sand is exposed by the tide. Then they are sealed in and have no access to oxygen outside the sand.

2. The concentration of dissolved oxygen in the sand water corresponds to a tension of about 7 mm. of Hg compared with a value about twice as great in the residual water in *Arenicola* burrows.

* Cf. the work on the respiratory function of chlorocruorin (Ewer & Fox, 1940), the dissociation curve (Fox, 1926, 1932) and the respiratory behaviour (Wells, 1951) of *Sabella pavonina*. This is a case where all three approaches indicate a high-tension oxygen transport system.

3. Extracellular pigments in the blood and coelomic fluid of *Nephthys* spp. are characterized as haemoglobins. The quantity of these pigments is shown to be inadequate as an oxygen store for the exposure period.

4. Dissociation curves for both pigments from *N. hombergii* are found to be approximately hyperbolic and the oxygen affinities relatively low.

5. The significance of the difference in oxygen affinity and the direction of the Bohr effect, between the vascular and coelomic pigments cannot yet be evaluated.

6. It is suggested that the *Nephthys* pigments are unspecialized and may function as a high-tension oxygen transport system only when the sand is covered by the sea. This is contrasted with the possibility of low-tension oxygen transport by the haemoglobin of *Arenicola*.

I wish to thank my colleague Dr F. Segrove for constant advice and encouragement and Dr Q. H. Gibson, Department of Physiology, University of Sheffield, for advice and helpful discussion on a number of physiological methods and for provision of sheep haemoglobin solutions of measured oxygen capacity. I am very grateful to Prof. E. A. Spaul for permission to make extensive use of the Marine Biological Laboratory of the University of Leeds at Robin Hood's Bay.

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SOME TEMPERATURE RESPONSES OF NYMPHS OF *LOCUSTA MIGRATORIA MIGRATORIOIDES* (R. & F.), WITH SPECIAL REFERENCE TO AGGREGATION

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INTRODUCTION

It is characteristic of locusts in the field that they form basking groups; in these groups hundreds of individuals collect in sunlit places and remain relatively quiescent. Poikilothermal animals generally show an increase of activity with rising temperature up to conditions that produce stupor, but the quiescence of basking locusts at high temperatures is not due to heat stupor, for the hoppers (nymphs) are still in a very reactive state, so that basking was for long considered as an anomaly. It was described by Kennedy (1939, 1945) as a negative thermokinesis superimposed upon the normal positive thermokinesis in insects moving in a close patchwork of temperatures. The group formation might be due to each individual reacting independently to the patchwork of temperatures, or it might be complicated by the insects reacting to each other. Ellis (1953), working on the interaction of hoppers in a uniform field, concluded that although mutual attraction did exist in older hoppers, the effects of simple physical features of the environment were more important. Such environmental effects have not hitherto been investigated in the laboratory, and the present work is an attempt to fill the gap to some extent. Gregarious hoppers of *Locusta migratoria migratorioides* (R. & F.) were used in the experiments.

EXPERIMENTS WITH A TEMPERATURE GRADIENT

Since it was apparent from field observations (Shumakov, 1940; Clark, 1949; Régnier, 1931) that hoppers of various locust species tended to remain stationary at certain places in a patchy temperature field, an attempt was made to determine whether or not they showed a restricted temperature preference. It should be understood that the word 'preference' is not used here to imply any conscious choice on the part of locusts, but to mean that a locust remained for a relatively longer period at one temperature than at any other. The only previous work in this field was that of Bodenheimer (1929) on *Schistocerca gregaria* Forsk., Parker (1924) on *Camnula pellucida* Scudder and Rubtzov (1935) on a number of Asian grasshoppers. Thus only Bodenheimer worked on what is generally regarded as a locust, and his experiments were not strictly comparable with the present ones on *Locusta* because he used large numbers of hoppers at a time instead of single individuals. The work of all the above authors was criticized by Gunn (1934) for lack of control of humidity and convection currents and for disregard of differences between air and floor temperatures.

Apparatus and methods

The apparatus used in the temperature-preference experiments was that used by Gunn (1934). The temperature at the cold end was maintained not by the method illustrated by Gunn but by a lagged box which surrounded the air inlet, and which could be filled with ice or water. In the experiments on first-instar hoppers, the box was ice-filled, but for later instars tap water was found to be sufficiently cold. A period of at least 3 hr. was always allowed for the gradient to become stable before any experiments were started. In this way, day-to-day variation in the temperature of the gradient was minimized. Thus, although the experiments were carried out in a cold room over almost a whole year the temperature at the cold end varied only from 9 to 11° C. for the first-instar hoppers, and from 16 to 21° C. for the later instars, while temperatures at the hot end varied only between 37 and 42° C. for all the instars. When it was observed that hoppers preconditioned at higher temperatures showed a higher preference, the temperature of the hot end was increased to about 45° C., varying from 41 to 45° C. The middle thermometers showed hardly any variation throughout.

Except in the case of fifth-instar hoppers in dry conditions, the air passing through the apparatus had in all cases a dew-point at 16° C., the air being passed through water before entering the chamber. For the experiment with dry air the water was replaced by calcium chloride and the dew-point was below 3° C., the lower limit of the apparatus used. The direction of air flow was reversed after each individual test, the rate of flow being 200 c.c./min. in each direction. The apparatus was arranged parallel with the only window in the room, which faced north, so there was no question of the hoppers being exposed to strongly directional light along the length of the chamber.

Hoppers were tested singly because of their tendency to aggregate in a uniform field (Ellis, 1953). Results obtained if large numbers had been used might have been due to such aggregation rather than a true response to temperature. Further, owing to the relatively small size of the chamber, it was not thought advisable to use more than one hopper at a time because artificial groups might have formed as a result of the size limitations of the enclosing space, particularly in the later instars. Bodenheimer (1929) found this to be the case and was forced to modify his experiments. In practice it did not appear that the size of the gradient influenced the movement of the single animals, even in the fifth instar, except, of course, at each end.

It was originally intended to follow Gunn's method of leaving each insect in the gradient until it had remained in one spot for a given time. Preliminary experiments showed that hoppers sometimes did not sit still for more than a few minutes at a time throughout a whole day, and also, when kept in for such long periods, they became progressively more active. The best method was found to be to keep each hopper in the chamber for only half an hour; it was then replaced by another. The position of the hopper in the chamber was plotted every minute, taking the position of the cervical joint for reference. The total time spent stationary at each temperature was summed at the end of a series of experiments and the results plotted as

histograms. In expressing the results it has not been considered advisable to attempt to show temperatures more accurately than at intervals of 5° C., since the head was often at one temperature and the abdomen at another.

Five thermometers were inserted into the apparatus through holes at equal intervals along the length of the gradient. Hoppers of the first three instars were introduced with equal frequency through each of these holes. No apparent disturbance of temperatures within the apparatus resulted from this method of introduction. Because of their larger size, hoppers of the fourth and fifth instars could not be introduced in this way, but they were put in equally frequently from either end of the chamber. The disturbance in this case was greater, but conditions became stable within a few minutes and the effect was, in any case, quite local.

Results

One hundred first-instar hoppers preconditioned over-night at 20° C. showed a peak at 30–35° C. in the histogram of time spent stationary (Fig. 1). A second peak shown at the lower end was due to the hoppers becoming physiologically trapped at temperatures below 15° C. Male and female hoppers gave similar results. Even hoppers less than 1 day old, of which fifty were tested, showed a definite tendency to remain longer at temperatures between 30 and 35° C. These hoppers had never before been subjected to temperatures in excess of 20° C. except during incubation of the eggs.

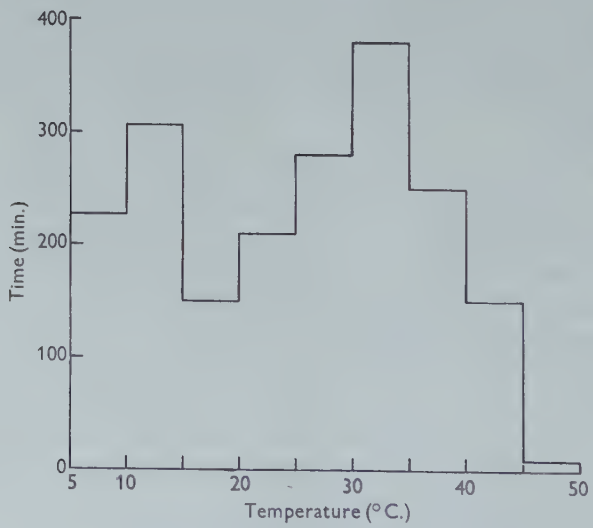

Fig. 1. Temperature preference of first-instar hoppers.

It was possible to find the rate of movement of the hoppers from their new starting point at the beginning of each minute. The results (Fig. 2), based on observations on 227 first-instar hoppers and not counting the time spent stationary, showed a steady increase in the rate of walking from 5 to 20° C.; above this the rate fell off. This latter fact was correlated with the inactivity of the hoppers from 25° C.

to nearly 40° C., and between these temperatures even those that were walking did so more slowly. The mode of recording was unsuitable for showing the increase in activity at temperatures over 40° C. because the hoppers became very excited and moved mainly by hopping instead of walking.

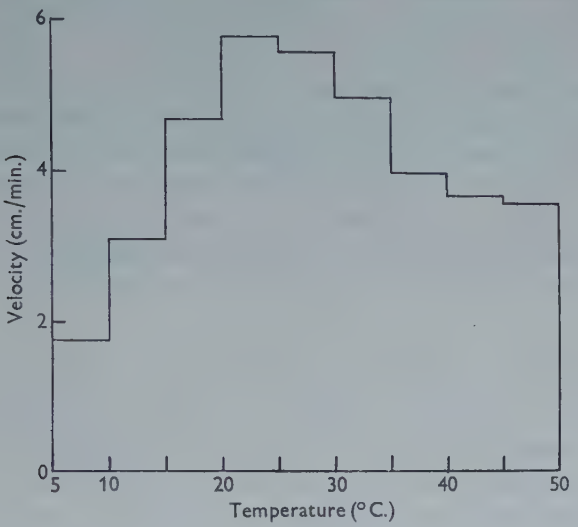

Fig. 2. Rate of movement of first-instar hoppers in the temperature gradient.

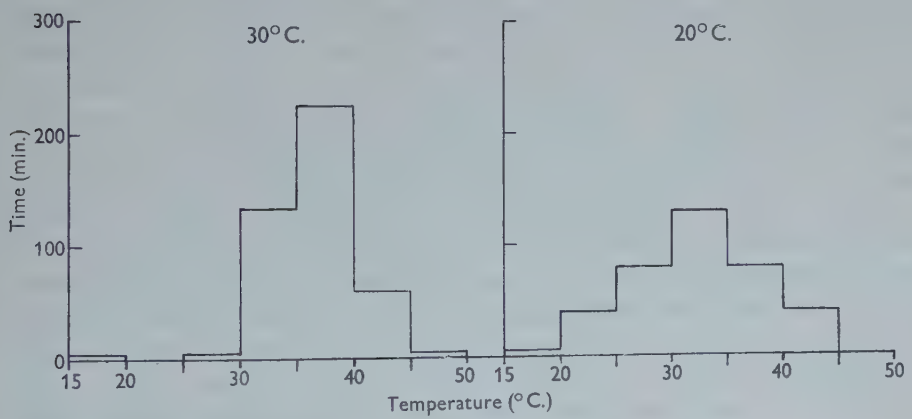

Fig. 3. Temperature preference of fifth-instar hoppers preconditioned at 20° C. and 30° C.

The later instars were tested in much smaller numbers, thirty hoppers of each being used. All the instars, when preconditioned at 20–25° C., showed a preference at 30–35° C. The fourth and fifth instars were also tested after preconditioning for some 3 hr. at about 35° C. In these cases, the range of temperatures at which hoppers remained stationary was much reduced. Thus fifth-instar hoppers only remained for 8 min. (less than 2% of the total time stationary) outside the range

30–45° C., whereas those preconditioned at 20–25° C. showed a normal distribution over the range 20–45° C. Furthermore, the hoppers remained longer at higher temperatures when the preconditioning temperature was higher, the peak in the histogram for preconditioning at 30° C. being at 35–40° C. (Fig. 3). For fifth-instar hoppers preconditioned at 20–25° C., the mean temperature was 32·4° C., while for those preconditioned at 35° C. it was 36·6° C. The difference was significant ($p < 0{\cdot}01$).

A comparison of fifth-instar hoppers preconditioned at 35° C. in wet and in dry conditions showed peaks in both cases at 35–40° C. The mean preferred temperature was 36·6° C. in the wet and 37·1° C. in the dry conditions; the difference between the two cases was possibly significant statistically ($0{\cdot}01 < P < 0{\cdot}05$), although only 0·5° C. Such a difference is of doubtful significance biologically, and it indicated that the range of humidity at any one time throughout the temperature gradient and due to it was not likely to influence the results to any large extent.

Discussion

The results obtained here are at variance with those of Bodenheimer who found that the temperature preferred by *Schistocerca* increased through the instars from 30·1 to 36·7° C. Herter (1923, 1924) claimed to have shown that in *Formica rufa* L., the preference increased with the external temperature, and Bodenheimer & Schenkin (1928) had similar results for a number of insects. Bodenheimer stated that the differences which he observed in *Schistocerca* were not due to this cause, but to differences in the physiological states of the insects. He did not give the preconditioning temperatures, but merely indicated the month of the experiment, which is inadequate. All these experiments are open to the criticisms raised by Gunn (1934) that floor and air temperature in Herter's apparatus, which Bodenheimer used, were different and that the latter varied with the external temperature. Since the thermometers in the apparatus were influenced by both air and floor temperatures, a small insect, reacting possibly to floor rather than air temperature, would appear to alter its preferred temperature in correlation with room temperature. This lays the results of Herter open to suspicion and may also account for the variation from instar to instar which Bodenheimer found. His difference between young and old adults might well be physiological. It has been shown here that for *Locusta* there is no substantial difference in preference from instar to instar with similar preconditioning, but that increased preconditioning temperature leads to a rise in the preferred temperature. Gunn & Hopf (1942), using entirely different methods, showed the importance of preconditioning temperatures in the temperature responses of *Ptinus tectus*. Gunn's temperature gradient is not open to the criticisms levelled at Herter's 'Temperaturorgel', and the hoppers were all bred under similar conditions prior to the preconditioning period of 3 hr. Preconditioning took place in a room separate from that containing the gradient, this latter room being thermostatically controlled to 19° C. Broadly speaking, the range of temperature preference found here for *Locusta* agrees well with that obtained by Bodenheimer for *Schistocerca*, being about 30–35° C.

It has already been remarked that the mean result in dry air was slightly higher than that in moist air, the difference being considered of doubtful biological significance. However, it might be expected that in dry air the temperature preference would be increased, since it was shown by Bodenheimer (1929) that the body temperature of *Schistocerca* at about 40° C. was 3 or 4° C. lower in dry air than in moist. Thus for body temperatures to be similar in the two cases, the preference, measured as the temperature of the apparatus, would be slightly higher in dry than in moist conditions. On the other hand, if the insect reacts positively to dry air (Kennedy, 1937) a higher preferred temperature might be expected in moist air (Gunn & Cosway, 1938). The small effect in the present experiments may be due to the two components acting in opposite directions.

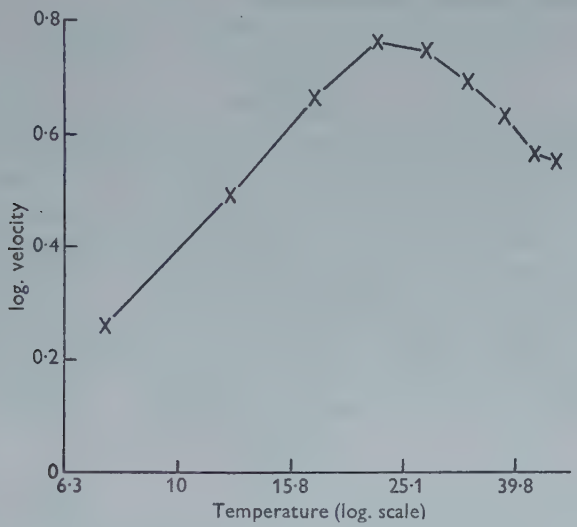

Fig. 4. Results shown in Fig. 2 plotted as logarithms, showing the applicability of Bělehrádek's formula.

The rate of movement of first-instar hoppers in the gradient was plotted as a logarithm against the logarithm of the temperature (Fig. 4). A straight line from 10 to 25° C. showed that the data for this range agreed closely with Bělehrádek's (1930) formula $v=\frac{a}{x}b$. The temperature coefficient for the range given and based on this formula is 1·06, showing a good agreement with Krogh's (1914) linear relation $yt=k$. Above 25° C., the curve, like the histogram, falls off.

These results are in general agreement with the views of Kennedy (1939) who stated (p. 504): '. . . negative thermokinesis, on theoretical grounds, could only be the dominant reaction in the field between 20 and 40° C. Below 20° C. weak positive thermokinesis would appear and above 40° C. it would supervene strongly.' The results given here provide quantitative data to support Kennedy's ideas for the lower temperatures, but they do so by using a different method of assessing activity. He used the proportion of time spent in motion, while here the speed of motion is

used. The manner of obtaining the results makes them unsuitable for showing the increase in activity at high temperatures because of the different form which the activity takes.

Bodenheimer (1929), Parker (1930) and Hussein (1937) studied the activity of locusts in a uniformly heated chamber the temperature of which was steadily changed. Under these conditions, all three authors found that the insects exhibited a continuous positive thermokinesis up to fatal temperatures. It is not obvious why there should be this difference between these results and those of Kennedy (1939) and the present work. The difference could be related to the post-prandial state of the hoppers, which, however, is not fully documented, or to the purely temporal temperature changes of the earlier experiments compared with the combination of spatial and temporal changes involved here.

EXPERIMENTS ON GROUP FORMATION

Apparatus

Twelve-litre cylindrical cages with metal bases and celluloid walls were used in the next series of experiments. The floor of such a cage was 20 cm. in diameter, and on it were drawn in chalk two concentric circles of 8 and 15 cm. diameter, giving inner, middle and outer floor areas in the ratio 4:10:11 approximately. By placing one of these cages on a trough of hot water (60° C.), 20 cm. in diameter, the whole of the bottom of the cage was uniformly heated. If, instead of a large trough, a beaker 6 cm. in diameter, filled with water at the same temperature as before, was placed centrally under the cage, the centre of the cage floor was warmed while the remainder was not, except for some conduction away from the centre.

Results

The tests were carried out with first-instar hoppers. In the 12 l. cages, hoppers normally climb the walls on the side towards the light. Counts of the distribution of the hoppers on the unheated floor showed that there was a marked edge-effect, the number in the outer area always exceeding the total number on the rest of the floor. This was not a sign of aggregation but was due to the wall-climbing.

Use of the large trough resulted in loss of the tendency to climb because of the more suitable temperature of the floor, so that the walls were almost entirely vacated and the whole population of the cage was on the floor. Again, a marked edge-effect was obtained because of the hoppers falling from the walls (Table 1). This was not true grouping and, although a tendency to group was sometimes observed, distribution of the hoppers was usually uniform except for the edge-effect. When the small beaker was used instead of the trough, a very marked group was invariably formed in the centre of the cage within 15 min. (figures in heavy type in Table 1). These groups were so dense on some occasions as to make accurate counting impossible. Similar results were obtained in complete darkness when no visual responses were possible (Table 2).

Discussion

These experiments made it quite clear that the formation of groups depended less on interaction between the hoppers than on the nature of the environment, hoppers collecting on the warmest available spots. Ellis (1953), working on the attraction of hoppers to one another, came to a similar conclusion. When in the present experiments there was a uniform temperature field, large groups were not formed, whereas the hot patch at the same temperature as the uniform field resulted in marked grouping. Hoppers, both younger and older than 3 days from hatching, were tested, because Ellis found no attraction between hoppers less than 3 days old. Groups were formed in both cases with the restricted temperature field.

Table 1. *Group formation in first-instar hoppers*

Normal				Floor temp. even				Floor temp. patchy				Age in days
S.	O.	M.	I.	S.	O.	M.	I.	S.	O.	M.	I.	
52	37	14	16	7	54	34	13	19	32	30	**36**	2
51	37	23	7	3	57	47	9	24	26	20	**40**	2
34	48	17	6	2	86	15	4	15	27	22	**47**	2
21	35	3	1	0	50	14	9	4	8	4	**50**	9
10	52	8	1	1	47	12	2	4	17	4	**46**	9
6	46	8	1	1	64	2	2	2	16	4	**50**	9
9	52	57	3	1	16	35	16	—	—	—	—	8
35	70	26	5	0	32	47	64	—	—	—	—	6
332	75	23	10	59	127	141	113	—	—	—	—	2
14	32	8	4	—	—	—	—	2	25	5	**44**	11
34	22	3	0	—	—	—	—	21	5	2	**35**	7
108	43	7	2	—	—	—	—	78	28	8	**28**	5
13	48	10	4	—	—	—	—	3	9	4	**59**	11
26	33	8	1	—	—	—	—	2	2	0	**64**	7
86	50	8	3	—	—	—	—	10	6	11	**120**	5
6	46	8	3	—	—	—	—	5	31	8	**19**	8
30	67	25	14	—	—	—	—	22	38	17	**51**	6
302	76	49	13	—	—	—	—	220	97	52	**71**	2
8	45	9	5	—	—	—	—	7	16	9	**37**	8
33	53	36	11	—	—	—	—	20	25	24	**64**	6
288	80	58	14	—	—	—	—	188	77	37	**138**	2

S.=sides, O., M., I.=outer, mid and inner areas of floor. Area O:M:I=11:10:4.

The experiments further suggest that, in the actual formation of the groups, it is the surface temperatures rather than body or air temperatures which are of importance initially. The hoppers might react to surface temperatures by way of sense organs on the tarsi or antennae (Geist, 1928).

BASKING GROUPS

The formation of basking groups in gregarious locust hoppers is a constant feature of the daily cycle of behaviour in the field, groups normally being formed in the morning and evening immediately after the descent from, and before the ascent to, the roosting sites. All field workers have indicated that basking takes place at temperatures below that at which marching occurs. Thus Allan (1933) found that basking in *Locusta* occurred below 82° F. (28° C.) and persisted all day if the air temperature did not exceed this, and that at higher temperatures the hoppers

marched. Predtechenskii (1935), working on *Schistocerca*, found that basking took place from 22 to 32° C. and marching from 32 to 40° C., and Fraenkel (in Bodenheimer, 1929) regarded 27° C. as about the temperature at which basking gave place to marching. Again in *Locusta*, Shumakov (1940) observed basking up to 43° C. and marching above this. Observations of other authors made it quite clear that basking groups were formed in the warmest available situations (Kennedy, 1939). Clark (1949) gave an example of basking above ground-level when the ground was cold due to recent rain. Records by Johnston (in Johnston & Buxton, 1949) of hoppers of *Nomadacris*, and by Régnier (1931) and Fraenkel (in Bodenheimer, 1929) of those of *Schistocerca* basking on stones and other bare surfaces exposed to the

Table 2. *Group formation in first-instar hoppers in darkness*

Normal				Floor temp. patchy				Age in days
S.	O.	M.	I.	S.	O.	M.	I.	
9	22	12	4	7	6	3	**48**	5
71	70	18	6	25	66	10	**64**	3
31	30	11	4	3	8	7	**58**	8
119	68	41	16	37	36	49	**122**	4
10	46	26	7	1	6	8	**74**	8
136	55	33	20	34	40	50	**120**	4

sun, may similarly be interpreted as 'choice' of the warmest available situations. The experiments on group formation described above were not comparable with basking-group formation in the field, since they were not carried out in the presence of radiant heat from above. Taken with the variability of the temperatures for basking given by other authors, they suggest, however, that group formation is not correlated with a particular temperature but with a patchy temperature field and the responses of the locusts to this situation. This was further investigated in a series of more natural experiments.

Apparatus

These experiments were carried out in a large cage 1·8 × 1·2 × 1·8 m. high. The walls were largely of zinc gauze with cellulose acetate observation windows and strips to prevent the hoppers climbing out, since the cage was open above. A source of radiant heat was present in the form of an electric heater with a parabolic reflector suspended about 1 ft. above the top of the cage. This apparatus will be described more fully elsewhere (Chapman, in press).

In support of the suggestions made above concerning the 'choice' of warm situations, tests showed that basking groups could be produced in any desired spot by placing a small piece of plywood on the hard-board floor of the cage, such pieces of wood being comparable, in their relative warmth, with the stones mentioned by field workers. Hopper temperature within such groups was about 35° C., which showed a good agreement with the temperature-preference experiments. Groups were formed only in places where a surface temperature exceeded the general floor temperature.

Some experiments were carried out to see if surface texture was of any importance in basking-group formation. Two similar pieces of hardboard 7·5 × 12·0 sq.cm. in area were grooved to take a thermometer, one on the rough side, the other on the smooth. The rough side was a little paler in colour than the smooth. These two plates were placed side by side in appropriate parts of the cage, each with a thermometer in position with the bulb in the groove so as to be just below the surface of the hardboard and obtain an approximation to the surface temperature. The column of mercury exposed was the same in both cases, and the thermometers were exchanged after each experiment to avoid instrumental error.

Results

In the absence of radiant heat, a series of counts of hoppers on the two plates of hardboard was made at half-hourly intervals throughout 3 hr. tests. In thirty-four such counts, the numbers on the two plates were equal on eight occasions, and in the remaining twenty-six tests the greater number of hoppers occurred on the rough plate on eighteen occasions. This difference was not significant ($p > 0.05$), showing that there was no marked preference for either of the surfaces. However, when the plates were subject to radiant heat, the greater number of hoppers was found on the hotter plate, irrespective of its surface. This was true in twenty out of twenty-one cases ($p < 0.01$). When the temperatures of the plates differed by 1° C. or more, the difference in numbers was most marked, groups being formed only on the hotter plate (Table 3, *a* and *b*). If the difference was less than 1° C. (*c* and *d*) groups formed on both plates but with more hoppers on the hotter one, except in one case where the converse was true. When the temperatures were equal (*e*), groups were formed on both plates and the larger number of hoppers might be found on either plate. These experiments showed that basking-group formation was dependent on a patchy temperature field, the hoppers being sensitive to difference of temperature of about 1° C.

Basking groups in the big cage were not static aggregations of hoppers but were in a very dynamic state. Their formation, persistence and break-up at a given site depended on the numbers of hoppers arriving and departing. Counts of hoppers arriving and departing from a group on one of the plates were made for 5 min. periods, at intervals over a number of 3 hr. tests with two bars of the heater on, so that ground temperature rose to a steady level of about 35° C. by the end of the first hour. Out of ten such counts in the first hour of the experiments, the number of hoppers arriving exceeded the number leaving in eight cases, but in the second hour the number arriving was greater in only three out of eleven cases (Table 4). In seven of the remainder the converse was true. Thus, over the first hour, basking groups were generally forming, while over the second hour they were breaking up. Counts obtained in the third hour were neglected because the hoppers were usually actively marching and counts became meaningless.

Alternating with the 5 min. counts described above, the time spent by individual hoppers in basking groups was also recorded. The average of twenty-four such readings was 6 min. 46 sec., varying from 2 sec. to 50 min. 56 sec.

Table 3. *Hoppers on the basking plates*

(*a*) Rough plate hotter than smooth

Rough (35·0° C.)	Smooth (34·0° C.)
10	4
18	3
25	5
34	6
26	1
36	2

(*b*) Smooth plate hotter than rough

Smooth (35·5° C.)	Rough (34·0° C.)
4	1
5	2
11	1
17	0
16	1
20	7

(*c*) Rough little hotter than smooth

Rough (36·0° C.)	Smooth (35·5° C.)
14	6
17	14
22	16
15	10
24	17
21	16

(*d*) Smooth little hotter than rough

Smooth (36·0° C.)	Rough (35·5° C.)
12	13
22	17
24	15

(*e*) Temperatures of two plates equal (37·0° C.)

Smooth	Rough
8	7
19	7
22	13
10	18
18	12
13	7

Discussion

Under the conditions described, groups were formed only on surfaces hotter than the general floor surface. There was thus no doubt that true basking groups did not differ from the groups formed in the 12 l. cages; environmental temperature differences due to radiant heat had the same effect as differences due to other causes. Hopper temperature in these cases was in the region of 35° C., but hoppers still

collected on the warmest available surfaces and did not aggregate elsewhere. This was in keeping with the observations of field workers outlined above, and further indicated that patchy surface temperatures must be of importance initially in the formation of basking groups.

Table 4. *The dynamic state of basking groups*

(*a*) First hour

	No. hoppers/5 min. period									
Arriving	4	3	7	9	11	17	6	11	7	9
Departing	1	3	5	5	9	16	4	12	3	5

(*b*) Second hour

	No. hoppers/5 min. period										
Arriving	7	2	8	4	3	6	8	8	11	14	9
Departing	8	6	12	5	2	6	11	9	5	17	7

The changes recorded in the groups in the preceding paragraphs were not associated with any changes in temperature, for by the end of an hour ground temperature was fairly steady at 35° C. They may be correlated with the physiological state of the hoppers. The observation that on some days hoppers at the end of a 3 hr. period of radiant heat were marching, while on other days, under identical external conditions, all would be basking, can also be explained on the basis of their physiological state. It is known that the amount of marching increases with the degree of starvation (Ellis, 1951), and Rubtzov (1935), having worked on a number of grasshoppers, stressed the importance of the 'previous physiological state' of the insects with regard to feeding, degree of desiccation and so on. Strel'nikov (1936) also observed that marching depended not only on the temperature of the hoppers but also on their 'general state of excitability'. These observations show that it is misleading to state that hoppers bask up to a certain temperature, above which they march and at which they resume basking later. A deciding factor is the physiological state of the hoppers. In so far as this is dependent on temperature, so will the temperature at which basking begins and ends vary.

SUMMARY

1. The results of experiments in a temperature gradient showed a definite temperature 'preference' on the part of hoppers (nymphs) of all stages. This 'preference' was constant from instar to instar but varied with the preconditioning temperature.

2. The rate of movement of first-instar hoppers was shown to increase in a linear manner with temperature up to 25° C., above which the rate fell off. It is suggested that these are quantitative data supporting Kennedy's (1939) remarks on negative thermokinesis.

3. Experiments in 12 l. cages showed that group formation depends on a patchy temperature field rather than on any particular temperature, and that environmental conditions are more important than mutual responses of the hoppers. Hoppers less than 3 days old, as well as older ones, formed groups under the conditions of patchy temperature.

4. The experiments suggested that surface temperatures are more important than air or body temperatures in the initial formation of groups.

5. Basking groups induced by local radiant heat in a large cage did not differ in form from the groups in the 12 l. cages formed in the absence of radiant heat.

6. Surface texture was shown to be unimportant in group formation, hoppers always collecting on the hotter surface even when temperature differences were of the order of only 1° C.

7. The groups were shown to be in a very dynamic state, with hoppers continually coming and going. The average time spent in a group by any one hopper was 6 min. 46 sec.

8. Formation of basking groups in the field depends on the physiological state of the hoppers, rather than on any definite temperature.

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HOMING ORIENTATION IN PIGEONS IN RELATION TO OPPORTUNITY TO OBSERVE THE SUN BEFORE RELEASE*

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INTRODUCTION

The aspect of the homing problem with which these experiments are concerned is the question of within how short a time birds are able to show homeward orientation after they are released in strange territory out of sight of familiar landmarks, and on what factors the speed of orientation depends. The sun navigation hypothesis which has been formulated by Matthews in various articles (e.g. 1951*a*, *b*, 1952, 1953*a*, *c*) suggests that the bird is able to discover the general direction of displacement from home by observing the position and movement of the sun and interpreting these in relation to the observations it made of the sun during its flights about the home loft. More specifically, the sun navigation hypothesis as put forward by Matthews requires that the bird observes the curvature of the sun's path during a period of time and extrapolates the sun's motion along the observed curve to infer the elevation of the sun at its highest point and the time of reaching it (local noon). The hypothesis assumes that the bird can infer whether it has been subjected to north or south displacement from the home loft from the altitude of the sun at its highest point. The bird is also assumed to have a highly accurate time sense and to be able to obtain an appreciation of east or west displacement by the difference between the time the sun reaches the zenith at the home loft and the time at which it will do so at the position of release. Matthews assumes that by the automatic or unconscious combining of these factors, the bird is able to direct its flight along the general course that would tend to restore the sun to its accustomed place in the sky, and by so doing ultimately reaches a familiar region in the neighbourhood of the home loft from which it can direct itself to the loft visually.

An obvious question raised by the sun navigation hypothesis is: How long is the interval of time during which the bird must observe the sun at the release point before it can show correct homing orientation? If this time is very short, this fact throws considerable doubt on the correctness of Matthews's explanation of how the bird might do this by sun navigation. Matthews transported his birds in opaque boxes, and each bird was tossed into the air immediately after being taken from its container. The orientation time was taken as the period from the beginning of flight until the bird vanished from sight as observed through field-glasses. Since

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the birds generally circled near the release point for about 3 or 4 min. before they moved off in the direction in which they finally vanished, Matthews considered that this initial delay in departure represented the time during which the bird was making the necessary observations of the sun and so gathering the essential information for showing homeward orientation. Kramer (1953) kept pigeons in the dark until 30 sec. before they were released, and he reports that these birds showed homeward orientation within 10 sec. after their release, or within 40 sec. of the time they were first allowed to see the sun. He found no appreciable difference between their orientation and that of birds with much longer exposure to the sun at the release point. Matthews (1953*a*) has pointed out that there was a difference in the accuracy of orientation between Kramer's two groups, the birds with previous sun exposure starting out, on the average, closer to the home line. He suggests, moreover, that perhaps even a 40 sec. period for observing the sun is sufficient for the bird to take account of the sun's position and apparent motion and to make the homing orientation response to them.

The initial period of circling was not found by Kramer in his birds, and it has not been found generally among our own birds. The difference between this behaviour and that found by Matthews may be the result of any of the following differences between the methods of handling the birds used by Matthews, by Kramer and by ourselves. Matthews's birds were kept in the darkness until the time of release and were therefore not light adapted when they first started to fly, whereas ours were exposed to the light before being released. Kramer's birds and ours were released from a high point (generally a forestry lookout tower), and they therefore had no need to circle in order to gain height and in most cases did not show any circling behaviour. Matthews's birds were confined in the home loft except for limited exercise periods, whereas Kramer's and ours were free to make flights about the home loft. Matthews's birds had been previously trained to fly along a course different from that of the home line at the time of the experimental release, whereas ours and Kramer's had no such previous directional training. Matthews's birds were thrown into the air, whereas ours were left free to look around before launching themselves on their flight.

Our main objective was to see if homeward orientation could be observed sooner than the 40 sec. interval after exposure to the sun reported by Kramer. The basic procedure for trying to shorten this critical time interval was one that was suggested by Kramer in conversation with one of us. This was to keep the release crate in the shadow of some opaque object so that the birds could become light adapted without being able to observe the sun. Thus, the time of exposure of the bird to the sun itself could be measured from the beginning of the flight.

PROCEDURE

During approximately 2 weeks prior to an experimental release, the pigeons were made accustomed to the crates and to the handling they would receive in the test; this will be referred to as the 'accustoming period'. On three or more occasions the birds were picked up in the loft at night, held in the crates until the forenoon of

the following day, and then released singly from a crate within sight of the loft at a distance of approximately 10 yards. Three sides and the top of each crate were constructed of wooden rods which admitted light or gave direct exposure to the sun when necessary. The fourth side of the crate was a sliding panel which could be removed to allow the bird to start its flight. During each accustoming period the pigeons were fed and watered in the crates. The accustoming procedure provided no homing experience of any kind, and, as the birds used in several of the experiments had never previously been displaced, their experimental releases were the first they had experienced out of sight of the loft. This procedure was adopted after Matthews (1953*b*) discovered that untrained pigeons may show accurate homing orientation at the release point, and after one of us (Pratt, 1955) confirmed this result and showed also that untrained pigeons can return home successfully in satisfactory numbers.

Before starting the journey to the release point, each bird was provided with a special message band requesting information in case the bird failed to reach home and was found.

Precautions were taken to ensure that the birds were transported under conditions that prevented their receiving any clues from the landscape or regarding the position of the sun during the journey. For most of the experiments the pigeons were hauled with the crates in the trunk of a car. The birds were covered with cardboard and canvas, and the lid of the trunk was raised slightly for ventilation. In two experiments, the crates were carried inside a large cardboard box on an open truck, and special ventilation holes were provided to admit only indirect light.

In some of the experiments comparisons were made between the orientation of birds with exposure to the sun before they were released, and of birds that were kept in the shadow until they flew. These two groups will be referred to as the 'sun' and 'no-sun' birds. The conditions during the transport were the same for both groups. After reaching the release point, however, the sun birds would be taken from the transport vehicle and the crate placed in the open so that it was exposed to direct sunlight for a period varying from 30 min. to several hours before the releases. The no-sun birds, on the other hand, were kept in the shadow through such precautions as parking the vehicle so that the opening through which the birds were removed was always in the opposite direction from the sun, and by carefully keeping an opaque object (a heavy paper bag or cardboard box) between the bird and the sun when it was being moved from the vehicle to the release crate, which was in the shadow of some opaque object.

Forestry lookout towers were used for the releases, and each experiment is identified by the official name of the tower used. The towers are open structures from 60 to 120 ft. tall. They are surmounted by a room with windows on all four sides. The cabin, which is equipped with an azimuth circle and pointer for taking bearings, is reached by several flights of steps connecting a series of platforms. The pigeons were carried up the tower in the crates, the no-sun birds being carefully covered and the crates being placed in a position where no direct sunlight could strike them. The release crates were placed on one of the platforms near the

top of the tower, the one for the no-sun birds being shaded by a large square of cardboard attached to the framing of the tower, or, when convenient, by the cabin overhead. Either a second crate on a lower platform was used to release the sun birds or else the single release crate was shifted far enough to put it in the sunlight. Successive releases of birds in the same group were made either by changing the direction in which the crate was facing, or else, when it was more convenient, by leaving the crate in the same position with the release direction forming an angle of 90° with the home line. When birds that were awaiting their turn for release were on the open platform, they were covered up with canvas while any bird was departing. Sometimes the birds were kept in the cabin of the tower where the waist-high steel walls ruled out the possibility of their observing any birds on their departures.

The method of observation used during the first experiment (Piney Mt.) was one that has become more or less standard. After each bird was released, the observers followed its course of flight with field-glasses until it vanished from sight, and the time from the start of flight until the birds reached the vanishing point was taken. Each observer fixed the place where he lost sight of the bird in terms of some landmark, obtained the bearing of this vanishing point from the direction finder in the tower, and drew from memory on a circular chart his impression of the course the bird flew while it was in sight. This method gave a specific result regarding the bird's orientation only after a minute or more, and any question regarding how much sooner the homeward bearing of a bird had been seen could be answered only on the basis of the observer's subjective impression. In later experiments, therefore, we adopted the procedure of taking the bearing of the pigeon at specified intervals of time after it had started to fly. In the second experiment (Wilson) these intervals were 10, 20 and 60 sec. In all the rest of the experiments, the intervals used were 10, 20 and 40 sec. with a final bearing being taken for the vanishing point. A 40 sec. interval was substituted for the 60 sec. interval used in the Wilson experiment because we discovered that some of the birds had already passed from sight before a minute had elapsed.

Each observer had the task of observing the direction of the pigeon from the tower at two of these times after release. The method adopted was that he noted some object in the landscape which was in line with the pigeon after the first time interval. He then watched the pigeon's flight until the end of the second time interval when he again made a similar observation of a recognizable landmark. He then took the bearing of the landmarks he had noted by means of the pointer on the fire tower. The times were controlled by one of the observers counting aloud in time with a metronome which had been adjusted to beat at intervals of 1 sec. The time after release at which the pigeon disappeared was determined by means of a stop-watch.

Homing success was checked at the loft by observing the time taken by the birds that returned on the day of release, and the number of birds that reached home on successive days. In comparison with the observations made at the release point the data on homing success were of relatively minor importance. With the general

procedure of using untrained pigeons for critical experiments on their first release, however, it is essential to get enough birds back to be able to breed stock for future experiments from birds of demonstrated homing ability.

RESULTS

A total of ninety-six separate releases were made from six different towers, Medoc being used on two different occasions. In three of the experiments, birds from two lofts located in different directions from the tower were released. Over the series of releases, an effort was made to select towers that presented a wide variety of geographical situations. The release points in relation to the lofts are shown in Fig. 1 and the geographical situation of each loft and tower is described briefly. The distances involved varied from 40 to 238 miles and the directions from the release points toward home were fairly well distributed around the circle.

The first question is whether there is any evidence of homeward orientation in the flight of the birds as they were observed at the release point. This may be answered for all of the experiments combined by observing how the vanishing points are distributed in relation to the home direction. Of the total of ninety-six birds released, vanishing points were not recorded in six cases, either because the birds perched in nearby trees or because they were lost from sight too soon when they flew behind the structure of the tower or took evasive action to escape a hawk. The vanishing points of the other ninety birds are shown in the upper graph of Fig. 2. (Sixteen birds released in the Wilson experiment are recorded in terms of the 60 sec. bearings as no effort was made to observe the vanishing points.) In the graph the vanishing point of each bird is represented by a unit radiating block which shows the 10 degree sector within which the bird was lost from view. Sixty-one birds vanished toward home, and twenty-nine away from home. This is a statistically significant favouring of the homeward half of the circle ($\chi^2 = 11{\cdot}38$, 1 degree of freedom, $P = 0{\cdot}0008$).

The second question is: How do the sun and no-sun birds compare in homeward orientation as revealed by the vanishing points? This comparison is shown in the lower two graphs of Fig. 2. The no-sun birds shown in the graph on the left have their vanishing points apparently more closely grouped about the home direction than do the sun birds. In fact, forty-one no-sun birds vanished in the home half of the circle and only thirteen in the opposite half, a highly significant favouring of the home direction ($\chi^2 = 13{\cdot}50$, 1 D.F., $P = 0{\cdot}0002$). The sun birds, on the other hand, made 20 vanishing points toward home and 16 away from home, the difference being statistically without significance.

The nearly significant difference between sun and no-sun birds cannot be interpreted as an indication that the latter showed superior orientation for the following reasons. Since the primary purpose of the experiments was to investigate the speed of orientation of the no-sun birds and not to make a comparison between these and the sun birds, there were several releases in which sun birds were not used; it may be that the general conditions affecting homing orientation were better on these days than on the occasions when birds of both groups were released. Also,

in the Piney Mt. experiment, we used six pigeons of unknown quality which were obtained from pigeon fanciers in Richmond, Virginia. Since our primary interest was in the orientation of the no-sun birds, these doubtful birds from Richmond

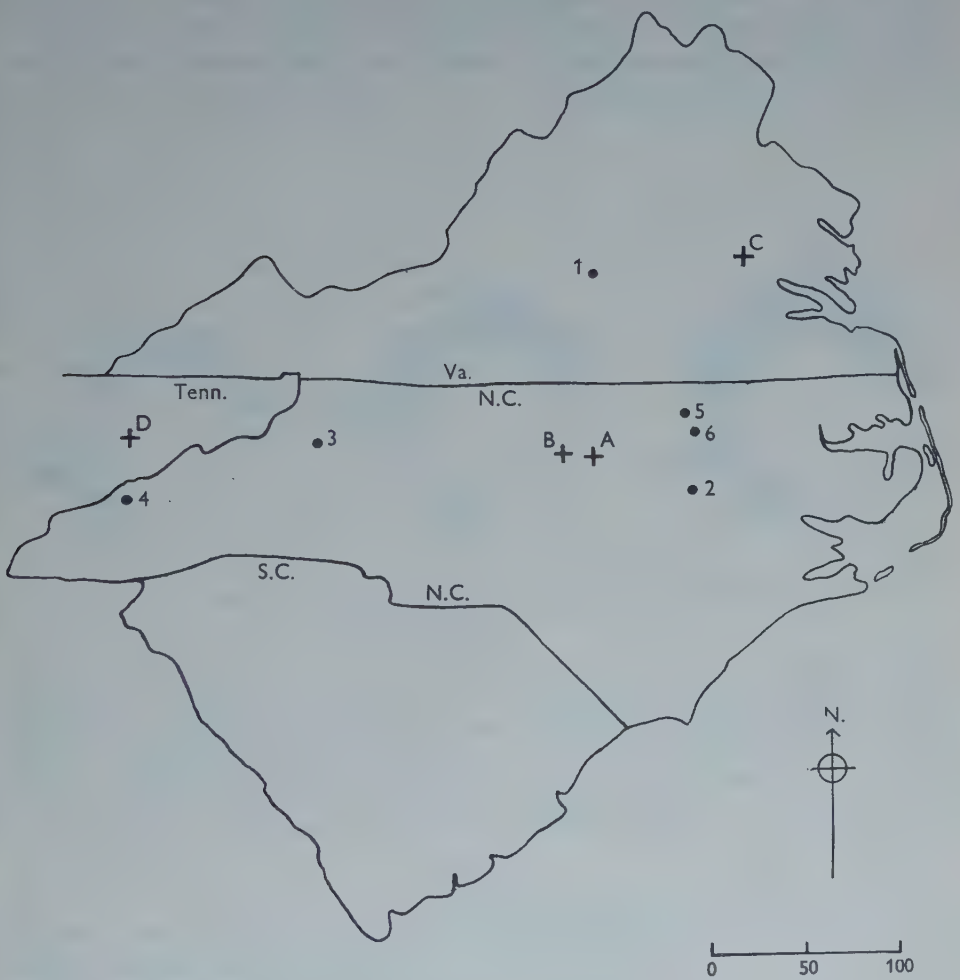

Fig. 1. Locations of lofts (lettered) and release points (numbered). A = Durham: loft in suburbs of city with prominent landmarks. B = Hillsboro: farm in hilly area of forests and fields two miles from small town. C = Richmond: residential section of large city. D = White Pine: loft at edge of small town near large lake. 1 = Piney Mt.: highest point on prominent ridge 4 miles north of Appomattox. 2 = Wilson: tower on western edge of a city similar to Durham. 3 = Dugger Mt.: high peak surrounded by mountains, no city in sight. 4 = Lick Stone: mountain peak with higher range toward White Pine. 5 = Warren: rolling coastal plain of forests and fields; water tanks 5 miles to north and north-west. 6 = Medoc: like Warren; nearest water tanks 12–15 miles to north, visible through field-glasses.

were all included in the sun group. If we consider only the birds released on occasions on which both sun and no-sun birds were used and omit the Richmond birds, there remain twenty-eight no-sun birds which disappeared in the home half

of the circle and twelve no-sun birds which disappeared in the other half of the circle. The corresponding numbers for sun birds were 17 and 12 respectively. A difference in proportions as large as this would occur on the basis of random sampling more often than 3 times in 10 on the average, so there is plainly no evidence which indicates a superiority of the no-sun birds and it is reasonable to suppose that their appearance of superiority in the total results is likely to have been due to the factors mentioned.

Fig. 2*a*: vanishing points for all releases combined. *b*: vanishing points for no-sun birds. *c*: vanishing points for sun birds.

The total figures given above for no-sun birds show clearly that these birds did show accurate orientation by the time they vanished from sight. It is, therefore, important to examine the data to see how soon after the start of flight (i.e. after the birds were first exposed to the sun) this orientation could be observed. This question can best be answered after the results of the individual experiments have been examined separately.

Piney Mt.

A total of thirty-one pigeons were released in this experiment, twenty-five from the Duke loft and six from racing lofts in Richmond. The Durham birds were about 18 months of age, and all of them were without any previous homing experience from the north, the direction in which they were displaced in this experiment. Six of them had homed from the south 6 months previously and some of the others had received short-distance non-directional releases 15 months before this release. We had a reasonable expectation of getting homing orientation at the release point in all the Duke birds except six that had been imported from Sumter, South Carolina, where they were hatched and kept in confinement until they were moved to Durham at 6 months of age. At the time of the experiment, these birds had flown free about the Durham loft for a year, but there is a widely held opinion among pigeon fanciers that birds do not home well to a loft to which they are moved after they are fully matured.

We had no accurate record of the flight experience of the Richmond birds, but considered that it was worth while using them as an indication of the extent to which departure flights were independent of local factors since no other controls for this purpose were available for this experiment.

Seventeen of the Durham birds were assigned to the no-sun group and eight to the sun group, the two groups being made as nearly equal as possible regarding stock and predicted homing ability. All six of the Richmond birds were assigned to the sun group. The distribution of the vanishing points of this experiment are shown in Fig. 3.

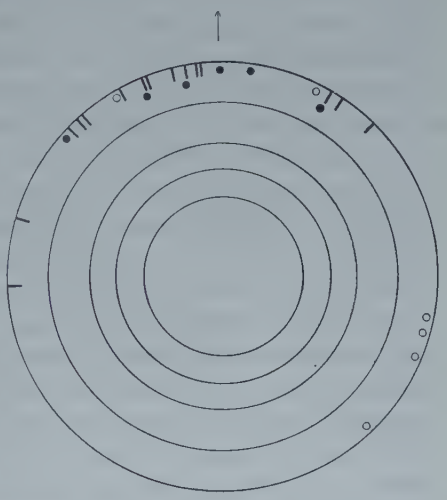

Fig. 3. Piney Mt releases. Only the bearings of the vanishing points were recorded. Birds: —, Durham no-sun; ●, Durham sun; ○, Richmond sun. Lofts: Durham, 100 miles, 183°; Richmond, 75 miles, 85°. Weather: wind, N.E. to E.N.E., 8–13 m.p.h. Clear. Temp. 55–70° F.

Three of the six birds imported from Sumter, South Carolina, into the Duke loft at 6 months of age landed in trees near the release point, and the other three circled within sight for several minutes. The rest of the Durham birds (and also the six Richmond birds) gave immediate departures with almost no circling. Since the orientation at specified intervals was not observed, it is only possible to compare the speed with which the sun and no-sun birds oriented themselves toward home on the basis of general impression and from the way in which each observer charted the bird's path of flight. As far as could be judged, there was no more initial indecision shown by the birds that were unable to see the sun until they started to fly than was observed in those birds with previous exposure to the sun. In a general way, therefore, this experiment confirmed Kramer's observation that the birds were already oriented toward home within 10 sec. of the beginning of flight, but it was the realization of the value of testing this impression on the basis of specifically timed observations that led us to change our procedure in later experiments.

Wilson

Sixteen birds were released in this experiment, eight of them being pigeons that had homed from Piney Mt. a week before, and eight of them being birds that had homed from the S.S.W. from 2 to 8 months previously. The Wilson tower was selected as one with a home line that bisected the angle formed by these two previous home directions. The main object of the experiment was to see whether the departures would be biased in the direction of the previous homing flights, and if so, whether the birds that had homed only a week before would show more bias than those for whom more time had elapsed since their previous homing flights.

As a matter of fact, when the general distance and direction of the release point had been decided upon, inquiries regarding the location of a tower that best met our requirements revealed that the Wilson tower was the most suitable on purely geometrical grounds. We recognized at once, however, that the fact that the city of Wilson was located within a mile to the east of the tower in the opposite direction from Durham might be a disturbing factor. It seemed probable that birds that had been accustomed to flying in the vicinity of Durham might be attracted by Wilson if they should mistake it for Durham. And it is even conceivable that any pigeons might be attracted by a nearby city. From the tower, Wilson looked to the human eye somewhat like Durham, with similar tall buildings and with aluminium water towers over factories and warehouses. As the question of the effect of local factors upon pigeon flight had already been raised and discussed by one of us (Pratt, 1953), we decided to proceed with the releases from the Wilson tower as an opportunity to gather evidence on this point. Nevertheless, the birds were divided into sun and no-sun groups, of similar composition as regards recent homing experience, on the prospect that some of them might show homeward orientation and thus provide information on the effect of exposure to the sun.

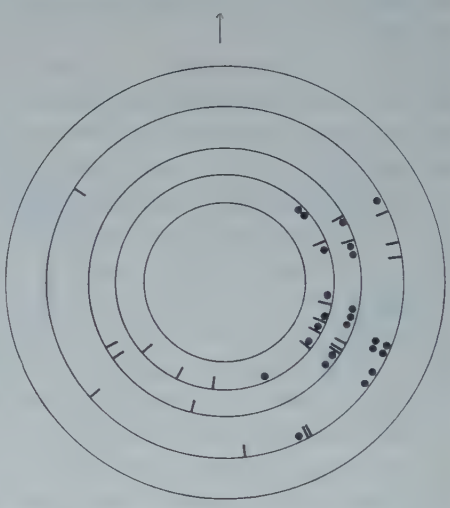

Fig. 4. Wilson releases. The bearings at 10, 20 and 60 sec. were recorded. Birds: —, Durham no-sun; ●, Durham sun. Loft: Durham, 58 miles, 288°. Weather: wind N. to E., 3–7 m.p.h. Small cumulus clouds, ground haze. Temp. 68–85° F.

The results (see Fig. 4) bore out our expectations that the birds might orient toward Wilson. Three observers were present for this experiment; one was assigned the task of recording the bearing of the bird after 10 sec., another after 20 sec., and the third at the 60 sec. interval. The tendency of the birds to fly toward Wilson or away from home is apparent at all three intervals. The suggested interpretation that the direction of flight was here determined by the attraction of the nearby city is, of course, only a tentative one which needs to be investigated further by comparing birds from a city loft with birds from a country loft when released in such a situation.

The lesson which seemed to emerge from the Wilson release was the possible importance of local conditions in determining direction of flight. The pigeons with their loft in the town of Durham showed a strong tendency, when released in the neighbourhood of the town of Wilson, to fly towards that city. It seemed, therefore, that the direction of flight may be determined by local conditions if these suggest to the pigeon a particular direction as the one in which it may most probably find its home. The most disturbing consideration is that if it had happened that the town of Wilson lay in the same line from the point of release as Durham the experiment

would have appeared to indicate the correct homing orientation. The possible influence of such purely local factors has sometimes been ignored in experimentation on orientation, but its importance is underlined by the result of the Wilson release.

One cannot, of course, get out of this difficulty merely by avoiding a release point in the neighbourhood of towns or other conspicuous objects. One does not know what visual marks will suggest to the pigeon the direction of home, and even a relatively symmetrical mountainous country or flat plain may contain indications visible to the pigeon which are not observed by the experimenter. It is also necessary to consider the possibility that the experimenter may select a release point which has wide open spaces in the home direction in order to provide an opportunity of observing their homeward flight for a long time. It is conceivable that the local features that are chosen as favouring observation may also affect the initial direction of flight.

The obvious way of overcoming this difficulty is to use a number of release points randomly selected in all different directions from the home loft. From the point of view of solving the problem of whether a pigeon shows homing orientation, each of these release points must be treated as a separate unit for the purpose of determining significance, and not each separate flight. A partial solution is to be found by releasing pigeons from different home lofts at a single release point and on the same occasion. We have used both of these procedures in the present series of releases, though not to the point desired for a final conclusion. The research will be continued at Duke University and it is hoped that eventually the facts about homing orientation can be established on the more rigorous basis of using the release point as the statistical unit.

Dugger Mt.

This experiment was planned to enable us to compare the homing orientation of sun and no-sun birds after specified periods of flight and to do so at a release point that took advantage of the lesson learned at Wilson. Twenty-eight pigeons of 4–6 months of age were used, none of them with any previous releases out of sight of the loft. Twenty of the birds were from a loft located on a farm 2 miles south-east of Hillsboro, North Carolina, and eight of them were from a control loft that had been established 1 mile west of the small town of White Pine, Tennessee. The Hillsboro birds were given the usual three nights in the crates with single releases from the release cage the following day, and the caretaker of the White Pine loft had been instructed to handle his pigeons in the same manner. The birds from White Pine were shipped by express on Saturday forenoon, reaching Durham on Monday about noon. They were confined in a small, semi-dark room for a day and a half and were crated again on Tuesday night together with the Hillsboro birds for the trip to Dugger Mt. Thus, the White Pine birds were either in the crates or confined in close quarters for a period of 4 days before the release, while the Durham birds were confined for only 15–18 h.

Dugger Mt., the release tower, was selected as a location almost directly on the line connecting the two lofts, and as a spot which was remote from any city or other

sign of civilization. The tower was a structure 60 ft. tall erected on a mountain peak with an elevation of 3333 ft. A range of higher mountains beginning approximately 4 miles to the west and running from S.W. to N.E. lay between the release point and the White Pine loft, and mountainous country with a lower elevation than the release point lay to the east and south in the general direction of the Hillsboro loft.

The results, in general, showed accurate and fairly immediate orientation toward the home loft in the departures of the Hillsboro birds, but less clear-cut evidence of homing orientation in the White Pine birds (Fig. 5). Whereas the Hillsboro birds flew without hesitation as soon as the crate was opened, the White Pine birds were reluctant to fly and they showed a great deal of circling after they left the crate.

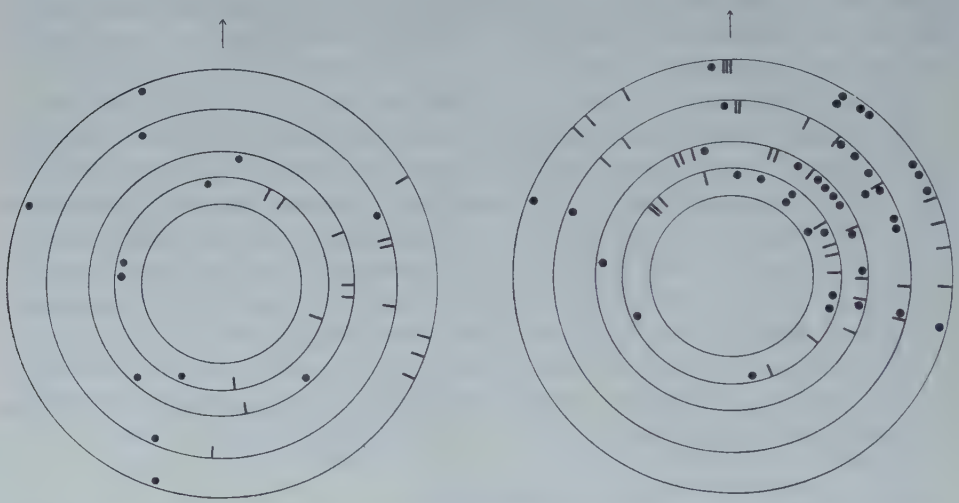

Fig. 5. Dugger Mt. releases. Bearings at 10, 20 and 40 sec. and at vanishing time are shown. Birds: no previous homing experience. Left-hand graph, White Pine loft. Right-hand graph, Hillsboro loft. Both graphs: —, no sun; ●, sun. Lofts: White Pine, 96 miles, 268°. Hillsboro, 140 miles, 92°. Weather: wind, N.N.W. to N.E., 4–10 m.p.h. Clear, ground haze. Temp. 45–60° F.

Two of them landed in trees nearby so that no observation of their orientation could be made, and one of them actually returned to a crate containing other pigeons on the tower and was picked up. In one sense, therefore, the White Pine birds did not provide a satisfactory control upon the homeward departures of the Hillsboro birds. But in another sense, they at least did not show any favouring of the half of the circle toward Hillsboro which was chosen by the birds from that loft. It is clear that the Hillsboro birds were not simply flying in the direction that any group of pigeons would have taken from this point. The evidence is strong, therefore, that the Hillsboro birds were orienting as they did because the home loft lay in that general direction.

Medoc

This tower was selected as one that was not in sight of any object such as a water-tank that might indicate a city. There were water-tanks at 10–15 miles to the north that could be seen through field-glasses, but these were approximately 90° off the

home line. Six birds were released on this occasion, three as sun birds and three as no-sun birds. Five of the six had made one previous homing flight toward the N.N.E. from 2 to 6 months previously, whereas the home direction from Medoc was W.S.W. The sixth bird had homed with a group toward the east up to a distance of 30 miles more than a year before. In view of the fact that the birds released at Wilson had not shown any effects of a recent homing experience but had reacted instead to local visual cues, we were not unduly concerned lest these birds released at Medoc should show a direction bias corresponding with their previous homing flight.

However, four of the five birds that had previously homed toward the N.N.E. did go off from Medoc in the same direction, all without circling ($Medoc_I$ in Fig. 8). One of the birds persisted in this direction as far as Richmond, a distance of 110 miles, where it was found in a racing loft the day after release. One of us had previously made observations which suggested that a single short distance flight may set up a direction tendency that persists for a few days. The present experiment suggested that a single long distance flight (75–150 miles) produced a direction tendency that was observable on the next release coming after a period of from 2 to 6 months. Thus, just as the Wilson release strongly indicated that local environmental factors might determine the direction of departure from the release point, so the Medoc experiment suggested that previous homing experience might be a disturbing factor strong enough to obscure any tendency toward accurate homing orientation. This is a possibility to be guarded against as far as possible in future work, but one that needs to be tested further as opportunities are found.

Lick Stone

This was a release on a small scale involving only three experienced homers from a Durham loft and three inexperienced pigeons from the White Pine loft. The Durham birds had all flown previously from the S.S.W., N. and E.S.E. In this instance, the Durham birds were hauled in the trunk of a car adequately covered to prevent their getting any information regarding the sun on a journey of approximately 300 miles to the Tennessee loft. They were kept there, still under cover, for one night during which the three White Pine birds were picked up and added to the crate. The following day a round-about journey through the mountains covering approximately 100 miles and taking 7 hr. was made in bringing the birds to the release point. This, like Dugger Mt., was a peak out of sight of any signs of habitation. As in the previous release, also, a range of high mountains lay between the release point and White Pine and there were unbroken mountains for about 40 miles in the Durham direction.

The departures from Lick Stone showed accurate homeward orientation on the part of the White Pine birds, but by only one of the Durham birds (Fig. 6). This suggests that the longer crating experience for the Durham birds may have been responsible, as we suspected might have been the case with the White Pine birds at the Dugger Mt. release. The only alternative suggestion that we can offer is that the Durham birds had made the flight both from Piney Mt. and from Wilson within

the preceding 3 weeks. It is conceivable that this release followed so closely upon the other two that the birds were either confused or discouraged in any effort to use their homing ability. The three White Pine birds showed accurate orientation. All six releases in this instance were done under the no-sun conditions.

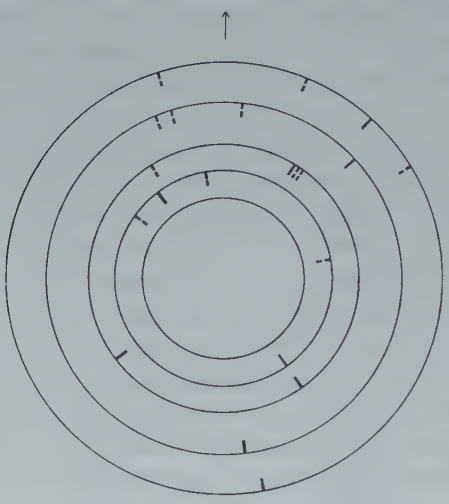

Fig. 6. Lick Stone releases. Birds: all no-sun. —, Durham; – –, White Pine (no previous homing experience). Lofts: Durham, 238 miles, 82°. White Pine, 40 miles, 348°. Weather: Wind, N. to E., 5–12 m.p.h. Clear. Temp. 45–60° F.

Warren and Medoc$_{II}$

The evidence that had been accumulated in the five sets of releases made had shown quite clearly that when the general conditions were such that the birds showed homeward orientation, this orientation was observable soon after the start of flight regardless of whether the birds had been exposed to the sun before they were released. The two groups of releases, however, at Wilson and Medoc had raised disturbing questions regarding the interpretation of homeward orientation in a number of birds set free at a single release point. In these two experiments the birds showed a consistency of orientation, but the preferred direction of flight was not that toward home. In these two experiments as well, however, there was no apparent difference between the orientation of the sun and no-sun birds. So if their inaccurate departures were cases of biased homing orientation, it seemed clear from the evidence that the choice of the direction of flight was not affected to a measurable degree by previous exposure to the sun.

Nevertheless, these two exceptional releases in which the birds flew away from home made it seem desirable to carry through in the time remaining for collaboration one further set of releases with inexperienced birds under the no-sun conditions. This was done with nine pigeons of the desired age, 6–8 months, that were available. In order to increase the number of release points used, five of the birds were turned loose at the Warren tower and the remaining four at the Medoc tower.

We were especially interested to make a second set of releases from Medoc to see if there was any tendency for inexperienced birds to fly off toward the N.E. as the first ones with previous homing flights in that direction had done.

Quite accurate and immediate homeward orientation was observed in the departures from both towers. From the results shown in Figs. 7 and 8, it is evident that, with only two exceptions, the homeward orientation was already observable after 10 sec. of flight, and it could be seen quite clearly by the end of 20 sec.

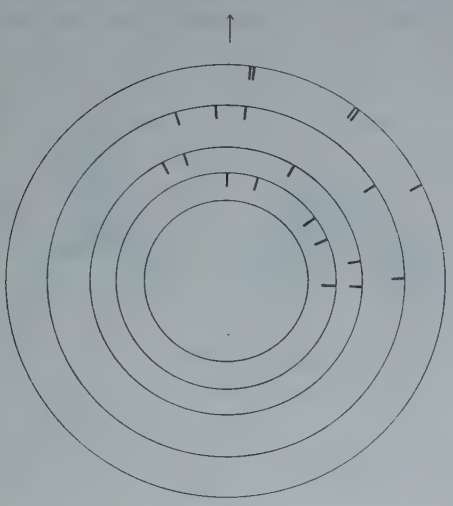

Fig. 7.

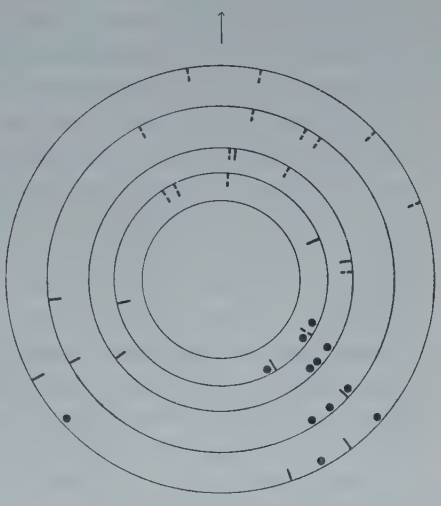

Fig. 8.

Fig. 7. Warren releases. Birds: Hillsboro, all no-sun. No previous homing experience. Loft: Hillsboro, 58 miles, 244°. Weather: Wind, S.S.E. 6 m.p.h. Clear. Temp. approx. 65° F.

Fig. 8. Two sets of releases at Medoc. Birds: $Medoc_I$: —, Durham, no-sun; ●, Durham sun. $Medoc_{II}$: – –, Hillsboro no-sun, no previous homing experience. Lofts: Durham, 59 miles, 253°. Hillsboro, 65 miles, 259°. Weather: $Medoc_I$: wind N. to E.N.E., 6–10 m.p.h. High fleecy clouds, ground haze, sun clearly visible. Temp. 55–70° F. $Medoc_{II}$: wind S.S.E. 6 m.p.h. Clear. Temp. approx. 65° F.

Flight orientation after 10 sec. exposure to the sun

With the details regarding the departure results of the separate experiments in mind, we are now able to consider the question of how soon the birds showed orientation after the beginning of flight and how this interval was affected by previous exposure to the sun. It has already been stated that accurate homing orientation was not observed in each set of releases, but the birds may nevertheless have been oriented almost from the start of their flight. As a general check on how soon orientation becomes apparent, therefore, we may consider the data from a somewhat different point of view than that which has been favoured heretofore. Let us take the bearing of the bird after a specified longer interval of 40 sec. (60 sec. in the case of the Wilson experiment) and then observe how far the bearing of each bird after 10 sec. deviated from this later observation. In other words, was the direction of the bird from the release point after 10 sec. any indication of

where the bird would be after 40 or 60 sec.? If it is found that the 10 sec. bearing is related to the 40 or 60 sec. bearing, is there any significant difference between the closeness of this relationship for the sun birds and the no-sun birds?

The results of all the departures in the releases in which the bearings were observed at specific intervals, permitting a study of how the 10 sec. bearings deviated from the later bearings at 40 or 60 sec., are shown in Fig. 9. It is evident from the two diagrams, one for no-sun birds and the other for sun birds, that the first 10 sec. of flight give a real indication of the direction in which the bird is going to continue to fly. Furthermore, the bearing after 40 or 60 sec. is a reliable indication of the bearing of the vanishing point. There were thirty-four instances in which the birds were in the home half of the circle at both of these times, thirteen cases when the bird was outside the home half of the circle at both intervals, and only eight instances in which the birds were in one half of the circle at 40 or 60 sec. and vanished from sight in the other half.

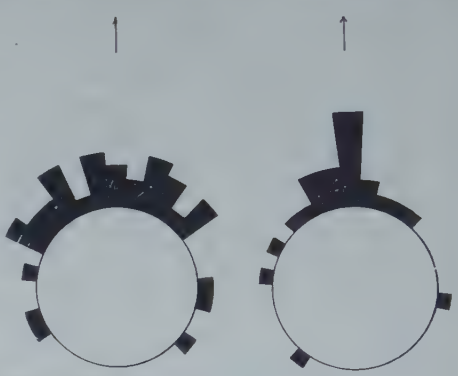

Fig. 9. Deviations of the 10 sec. bearings from those observed in each bird after 40 sec. (60 sec. for the Wilson release). Left, no-sun birds; right, sun birds.

There is a slight indication that between 10 and 40 sec. (or 60 sec.) the sun birds deviate from the initial bearing less than do the no-sun birds. The difference between the two groups is not statistically significant, however, and it may therefore be considered a sampling variation. There is a possibility that it may indicate a genuine orientation effect, but one that has no relevance for the sun and no-sun comparisons. A larger proportion of the sun birds were included in releases in which orientation was observed when it was not toward home. It is conceivable that oriented flight that occurs on some other basis (e.g. vision or memory) may be even more immediate and unchanging than genuine homing orientation. In either case, the orientation must have occurred earlier than 10 sec. if the direction from the tower at 10 sec. was related to the chosen course of flight.

Homing success

The question of the number of the birds returning to the loft and the time they took for the journey is not directly relevant to the problem with which we were primarily concerned. Nevertheless, the homing success is of interest, both as scientific information and from the point of view of judging whether the experimental procedure is a practicable one.

During the past five years there has been a rapid increase in experiments dealing with the homing problem. The pigeon has chiefly been used as the experimental animal, and unexpected findings regarding the homing ability of this species have followed quickly one upon another. Matthews (1951*b*) started his research on the assumption that pigeons had to be given several releases at increasing distances in

one direction before they could home from a distance. Kramer & St Paul (1952) showed that directional training was unnecessary, but that pigeons could choose the home direction at a distance of 200 miles after they had received several non-directional preliminary releases at a distance of about 10 miles. Matthews (1953*b*) then showed that it was not necessary to use any short-distance preliminary releases. Pigeons for which the first release out of sight of the loft was at a distance 50 or 75 miles showed homeward departures. However, Matthews's untrained birds showed a very low level of homing success, only one pigeon out of thirty-nine released returning to the loft. Pratt (1955) also found that untrained pigeons departed in the direction of home, but, in contrast with Matthews's results, approximately 50% returned from first releases at a distance of 75 miles. However, there was a significant difference in homing success between different strains of pigeons used.

Ideas regarding methods have also been changing rapidly, and so far no basic procedure of handling pigeons up to the time of a test release has been accepted as the standard. In these experiments we used the procedure of dispensing with short-distance preliminary releases altogether. The returns from our joint experiments were only 40% of the birds released (see Table 1). The fact that this is somewhat lower than the previous rate of homing success with untrained birds is not surprising, as our releases were made chiefly with the Duke stock which previously was shown to be less successful in returning to the loft. The stock that was outstanding in homing ability was held for breeding.

Table 1. *Homing success*

Release and loft	Distance	No. of birds	Returns						
			Day of release	Second day	Third day	Fourth day	Fifth day	Later	Total
Piney Mt., Richmond	75	6	1	3	—	—,	—	—	4
Piney Mt., Durham	100	25	3	4	1	—.	1	2	11
Wilson, Durham	58	16	1	3	2	—.	—	1	7
Dugger Mt., White Pine	96	8	—	—	1	—.	1	1	3
Dugger Mt., Hillsboro	140	20	—	—	—	—.	—	1	1
Medoc, Durham	59	6	—	—	1	—.	—	1	2
Lick Stone, White Pine	40	3	—	2	1	—.	—	—	3
Lick Stone, Durham	238	3	—	—	—	—.	—	1	1
Warren, Hillsboro	58	5	1	1	1	—.	—	—	3
Medoc$_{II}$, Hillsboro	59	4	—	3	—	—.	—	—	3
Totals	—	96	6	16	7	—	2	7	38

In spite of the losses, we think that the method of using untrained birds for homing experiments is a practicable one and that for most research purposes it offers more advantages than using directional training or preliminary short-distance releases. The latter two methods also result in losses; and when these occur in the preliminary stage they are more costly, because they are suffered without gaining any information on the homing problem. With the method of using completely untrained birds, the release point observations of some of the birds that fail to reach home show evidence of homing orientation in their departures and the others may throw some light on the problem by their failure to start toward home.

We found in our first-flight birds a direct relation between the distance to be covered and the proportion of birds lost. In the work now being done at Duke University, therefore, the first release is still being made for test purposes but at a relatively short distance. This procedure serves several purposes. First, and most important, it cuts down on the losses from the first release. Secondly, it will yield results bearing on the question of the basis of short distance orientation. Thirdly, the birds can be used in later releases at greater distances with the prospect of fewer losses.

DISCUSSION

Until recently it was commonly reported that homing pigeons circled for a short period or flew at random near the release point before they oriented toward home. In the recent work at the Max-Planck-Institut in Wilhelmshaven, Kramer and his associates found that their pigeons did not circle, but showed homeward orientation within a few seconds after release. The procedure used at Duke University was modelled after the German investigations and gave the same results: typically, the birds did not circle in their initial flight. This fact opened the way for the present experiments to find out how soon after release homing orientation could be observed and whether exposure to the sun before release made this time any shorter.

Heretofore, investigations have recorded the vanishing point and the vanishing time of each bird at the release point. We recorded the vanishing points for seventy-four releases and the 60 sec. bearing for sixteen others, and when these are pooled for the seven experiments they show a significant favouring of the home direction. The instances of departure in the wrong direction were not evenly distributed throughout the series of experiments, however, but occurred chiefly on two occasions. We feel fairly confident that we understand why the birds turned away from home in the Wilson release (false recognition of a nearby city), and we were able to suggest an explanation of the deviation in the first Medoc release (direction of a previous homing flight). Nevertheless, the tendency for pigeons released singly to agree on a direction of flight on each occasion, whether it was toward or away from home, emphasized a need for caution in regarding each flight as an independent observation for purposes of interpreting the departure results. This difficulty might be overcome by using a large number of release points and considering the mean deviation of the birds from the home line at each release point as the statistical unit, or it might be sufficient if birds with their lofts in different directions from a release point were shown to choose different directions that were related in each case to the distant lofts. In the present series of experiments we used both types of controls, employing a number of release points and, when possible, releasing birds to fly in two directions, but we did not carry this to the point required for a final conclusion. The findings are offered as only tentative until it is known that they are confirmed.

Even with this reservation, however, the results give strong indications that birds are capable of choosing the direction toward home within 10 sec. after they see the sun. If this is a fact, it weighs heavily against any hypothesis requiring observation of the sun's apparent motion as the basis of homing orientation. The prospects of

reaching a definite conclusion by further work along the lines of the present experiments seem good; and while the effort required will be considerable, it is not prohibitive. The difficulties are not restricted to the questions with which this research was concerned, but are inherent in all homing investigations using the methods now current. An advance in procedure which is badly needed is a way of studying homing behaviour without actually releasing the pigeon. Different investigators have attempted to do this, but thus far none of the efforts have met with success.

SUMMARY

1. A total of ninety-six single releases of pigeons was made over distances varying from 48 to 238 miles. The birds were transported under cover which prevented them from seeing the landscape or the sun. It was generally their first release from a point out of sight of the loft.

2. The vanishing points (as observed through field-glasses) from all releases combined showed a significant tendency to be in the homeward half of the circle.

3. The direction of the bird from the release point was observed at various intervals of time from 10 sec after release. There was a statistically significant relationship between the direction at 10 sec and that at 40 sec (or 60 sec.), and the latter directions were closely related to those of disappearance.

4. To test the hypothesis that correct orientation depends on an interval of time for observing the sun's motion, some birds were allowed to see the sun for some time before flight, while others were kept in shadow. Even after the short interval of 10 sec., the no-sun birds showed no less accuracy of orientation than did the sun birds.

5. In two releases the pigeons showed a consistent choice of direction not within the home half of the circle. This suggests the need for caution in drawing conclusions based on few release points, and the desirability of using many release points and taking the average orientation at each as the statistical unit.

6. Our results strongly suggest that there is no difference between the orientation of pigeons that have and pigeons that have not seen the sun before release. This conclusion is, however, tentative until it is confirmed by further experiments using a larger number of release points.

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THE BEHAVIOUR OF *ANODONTA CYGNEA* L., AND ITS NEUROPHYSIOLOGICAL BASIS

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INTRODUCTION

Apart from one paper (Nadort, 1943) dealing with some somatic reflexes in *Anodonta cygnea* and *Unio pictorum*, the only references to the behaviour of *Anodonta* seem to relate to isolated observations made by workers interested in other aspects of the physiology of these animals.

Pawlow (1885), in a paper demonstrating the existence of a double innervation of the adductor muscles, mentions both reflex and spontaneous adduction of the valves, and adds the remark that occasionally the spontaneous movements assume a regular periodicity. In 1906, Marceau showed that rhythmical activity of the adductor muscles was exhibited by many lamellibranchs of widely differing structure and mode of life. This rhythmical activity comprised alternate rapid adductions and slow abductions of the valves, the amplitude of the movements tending to decrease until the valves came to rest, in some genera, in the closed position, and, in other genera, in a widely gaping position. *Anodonta* was one of the former group. In a later paper (1909), he reproduced some kymograph records which demonstrated that in *Unio* (and, less obviously, in *Anodonta*) this rhythmical activity was merely part of a much slower rhythm, composed of alternate periods of activity and quiescence. He did not mention this, however, in the text of his paper. Marceau did not investigate the mechanism controlling these rhythms, nor did he offer any explanation of the function of the muscular contractions other than the rather strange teleological one: 'Ces conditions de distension constante, avec contractions et relâchements intermittents, sont nécessaires à la vie des muscles adducteurs, et le grand principe de physiologie générale "tout organe qui ne travaille pas s'atrophie" trouve là une éclatante confirmation.'

Koch & Hers (1943) have studied the movements of the siphons in *Anodonta*, and have shown that, during the animal's period of activity, the apertures are alternately open and closed, thus causing intermittent respiratory currents. When the animal first opens its shell valves after a period of quiescence it has incurred an oxygen debt, but as this debt is paid off the periods of flow of water through the siphons become progressively shorter and less frequent. The movements of the siphons may, therefore, be regarded as subject to a rhythm which is modified according to the respiratory needs of the animal. As far as one can tell from their kymograph recordings, there is no correlation between this rhythm and the adductor rhythm.

In his paper of 1943, Nadort first reviews the literature on the nervous systems of lamellibranchs, and then goes on to describe the method of locomotion and also

several reflexes found in *Anodonta*. As a result of experiments involving the extirpation of ganglia, he draws conclusions as to which of the ganglia are responsible for controlling these various activities.

The behaviour of *Anodonta* may be considered as composed largely of (*a*) activity of the adductor muscles, producing movements of the shell valves, (*b*) activity of the mantle margins, and of the siphons in particular, and (*c*) activity of the foot, resulting in locomotion. In so far as these activities are under nervous control, they could be induced either by stimulation of receptors (i.e. reflexly) or by 'spontaneous' functioning of the nervous system, or by both factors. The purpose of this paper is to describe experimental work on (*a*) which points to the importance of both factors in the neurophysiological basis of behaviour in *Anodonta*. A preliminary account of some of this work has already been published (Barnes, 1952).

MATERIAL AND METHODS

Most of the experiments described in this paper were carried out on large specimens of *A. cygnea* L., but in a few experiments where thick-shelled animals were desirable *A. anatina* L. was used instead. The latter experiments were, however, repeated with *A. cygnea* to ensure that any conclusions drawn from the experiments were equally applicable to this species.

The animals were kept in running water in laboratory sinks until required, and, although the specimens used for experiment were collected fresh every few weeks, some specimens were kept in an apparently healthy condition in this way for many months; their behaviour after nearly 4 months captivity was, as far as could be seen, identical with that when they were freshly collected.

For the purpose of recording the movements of the shell valves, the animal was placed on its left side with its valve embedded in low melting-point paraffin wax in a dissecting dish, leaving its right valve free to move about the axis of the hinge. A piece of thread fastened by means of sealing wax at some suitable point on the surface of the right valve was attached to a frog heart lever which wrote on the smoked surface of a kymograph drum. The animal could then be covered with still, running, or aerated water. The contractions of the two adductor muscles could be recorded separately by cutting across the right valve from the mid-point of its margin to the mid-point of the hinge, and attaching the two portions to two separate levers. Operations on the nervous system were performed by inserting instruments through small windows cut at appropriate places in the right valve. When it was desirable to exclude water after the operation, the windows were 'glazed' by means of cover-slips fixed in place with stiff petroleum jelly. The actual recordings were made on one or other of (*a*) a standard physiological kymograph, giving a range of speeds from 1 rev./sec. to 1 rev./hr. approximately, (*b*) an improvised kymograph, driven through gearing by a government-surplus electric motor, and controlled by a variable resistance to give a continuous range of speeds from 1 rev./4 hr. to 1 rev./day, and (*c*) a Casella barograph drum, giving 1 rev./day or 1 rev./week.

RHYTHMICAL MOVEMENTS OF THE VALVES

During the course of this work, the movements of the valves of sixteen intact specimens of *A. cygnea* have been recorded, each for a period of several days, so that the recordings cover more than 200 specimen-days. All of these recordings demonstrate the same type of behaviour. The animals spend alternate periods in activity and quiescence.

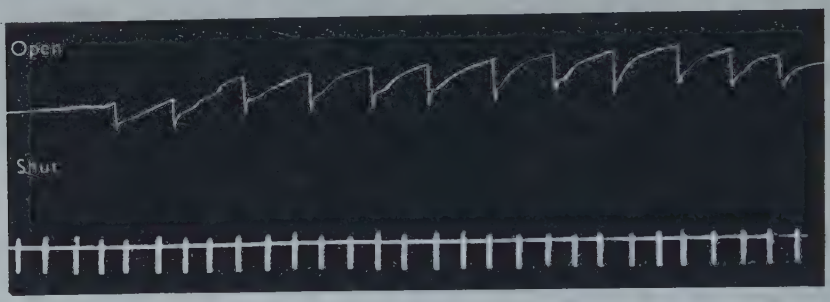

Fig. 1. Normal behaviour. Part of one period of activity. Time marker, 2 min. In all figures, recordings run from left to right, with contractions (adductions) downwards.

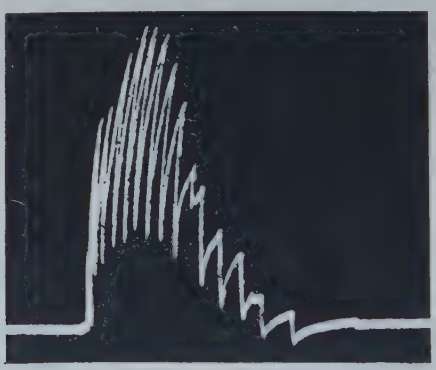

Fig. 2. Normal behaviour. The whole of one period of activity. Total time, 3 hr.

During the period of activity the shell is typically open, with the ventral margins of the valves separated by about 2–6 mm., but from time to time there occurs a rapid adduction of the valves followed almost immediately by a slow separation. These movements are repeated at intervals usually sufficiently regular to leave no doubt that one is witnessing truly rhythmical activity (Fig. 1). At its commencement the rhythm has (at room temperature) a frequency of approximately 12–20 adductions/hr., but towards the end of a period of activity the rhythm slows down. The adductions are not strong enough to lead to complete closure of the shell, but they are rapid enough to produce a vigorous jet of water from the exhalant siphon. The end of the period of activity is marked by progressively less complete abductions and stronger adductions, until the valves meet along their margins (Fig. 2).

Throughout the period of quiescence the shell is kept closed, with its valve margins held firmly together, but, if the thread to the recording lever is attached over the insertion of the posterior adductor muscle in a thin-shelled specimen, small rhythmical kinks in the recording indicate that the rhythmical activity of that adductor muscle has not ceased with the complete closure of the valves. In most instances the retardation of the rhythm, already noticeable during the active period, leads sooner or later to a cessation of these kinks during the quiescent period. In a few instances, however, the rhythmical kinks have persisted until the commencement of another active phase, when the frequency has again increased (Fig. 4A). The new active phase is initiated by increasing relaxation of the adductor muscles, causing the valves to gape, and allowing the rhythmical adductions to take place.

The alternate periods of activity and quiescence themselves conform to a rhythmical pattern, which, however, is not so regular as the more rapid rhythm (Fig. 3). The periods of activity are usually shorter than the intervening periods,

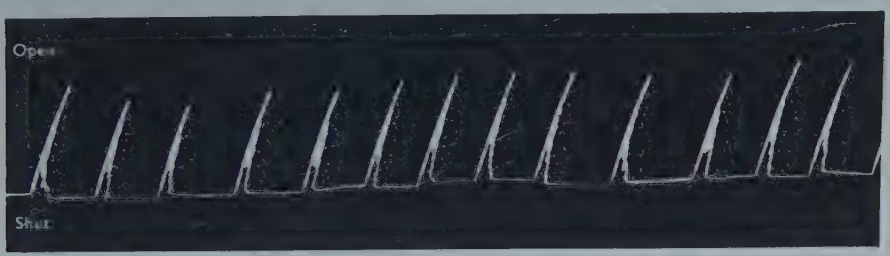

Fig. 3. Normal behaviour, showing alternate periods of activity and quiescence. Total time, nearly 3 days.

and they recur with frequencies ranging from 3 to 30 per week in the specimens investigated. Some of the irregularities in the rhythm are attributable to internal, and some to external, factors, e.g. (*a*) the occasional protrusion of the foot (particularly in well-aerated and agitated water), causing a great extension of the active phase possibly for a day or two; (*b*) considerable variations in the temperature of the laboratory, which are likely to influence the frequency significantly (Pawlow (1885) observed a marked change in the rate of activity of the animal over a temperature change of 3–4° C.); and (*c*) unavoidable vibrations, which have been observed to induce reflexly the commencement of an active period (see p. 171). But despite the irregularities the behaviour of the specimens investigated conforms to such a consistent pattern as to leave no doubt that it is rhythmical.

For the sake of brevity the two rhythms described above will hereafter be termed the 'rapid' and the 'slow' rhythms respectively.

THE NEUROMUSCULAR MECHANISM OF THE RHYTHMS

If the valve which is free to move is cut across from the mid-point of its ventral margin to the mid-point of the hinge, and simultaneous recordings are made of the movements of the two halves, it is found that both adductor muscles participate in the rapid rhythm. In order to demonstrate this satisfactorily, however, it is usually

necessary to weight the anterior lever to abduct the valves between contractions, since the elastic ligament of the hinge operates only on the posterior part of the shell. If these recordings are continued it is found that both portions of the cut valve are after a few hours fully adducted, and that they remain fully adducted until the animal becomes moribund after a week or two. It is therefore impossible to ascertain by this means whether both adductor muscles are concerned in the slow rhythm as well as the rapid rhythm. But this experiment does indicate that both muscles are, at least, capable of maintaining a strong contraction over a long period of time. Whether they both in fact do so during the period of quiescence has to be determined by other experiments. The most satisfactory method is the following.

An animal with a thin and therefore pliable shell was selected and a window of about $\frac{1}{2}$ in. in diameter was cut into the right valve in the region of the shell's greatest bulge, so that when the specimen was fixed in the dissecting dish in the usual manner the window was at the highest point of the shell. Water was then poured into the dish until it rose to a level just below the window. The animal continued to show its normal type of behaviour. Then during a period of quiescence a fine scalpel was introduced through the window and the posterior adductor muscle was cut from its insertion on the upper valve. Immediately the valves separated by about 3 mm. at their posterior end, while at the anterior end they remained in close contact. This result indicated that before it was cut the posterior adductor muscle was responsible for keeping the valves together by its contraction. Another experiment was then set up to test the effect of cutting the anterior adductor muscle in the same way, but, as the elastic ligament operates only on the posterior end of the shell and would be counterbalanced by the contracted posterior adductor muscle, it was necessary to employ a means of abducting the anterior end of the valves so that the effect of cutting the anterior muscle could be ascertained. For this purpose a thread attached to the upper shell above the insertion of the anterior adductor muscle was tied at its other end to the hook of a spring balance, which was suspended vertically from a movable clamp. The clamp was adjusted so that a tension of 100 g.-wt. (a small tension compared with that exerted by the elastic ligament)* was recorded by the spring balance when the valves were completely adducted. This did not interfere with the normal rhythmical activity. When the anterior adductor muscle was cut during a period of quiescence the anterior end of the shell gaped by about 2 mm., the spring in the balance contracting, of course, by the same amount. Thus the anterior adductor muscle must also be in a contracted condition during the quiescent phase of the slow rhythm. During the period of the animal's activity it is obvious that both adductor muscles are relaxed except for the periodic contractions of the rapid rhythm. So it may be concluded that both anterior and posterior adductor muscles exhibit the same type of activity, both participating in the rapid and the slow rhythms.

Marceau (1909) showed that the adductor muscles of *Anodonta*, like those of many other bivalves, are composed of two portions, one consisting of unstriated fibres

* A mass of 450–600 g. placed over the same region of a completely eviscerated shell is normally required to bring about its complete closure against the tension of the elastic ligament.

capable of maintaining a tonus for long periods of time, and the other consisting of spirally striated fibres capable of rapid contractions but incapable of maintaining a tonus. It seems probable then that the movements of the rapid rhythm are brought about by the phasic fibres, while the slow rhythm is explicable in terms of increased and decreased tonus of the tonic fibres.

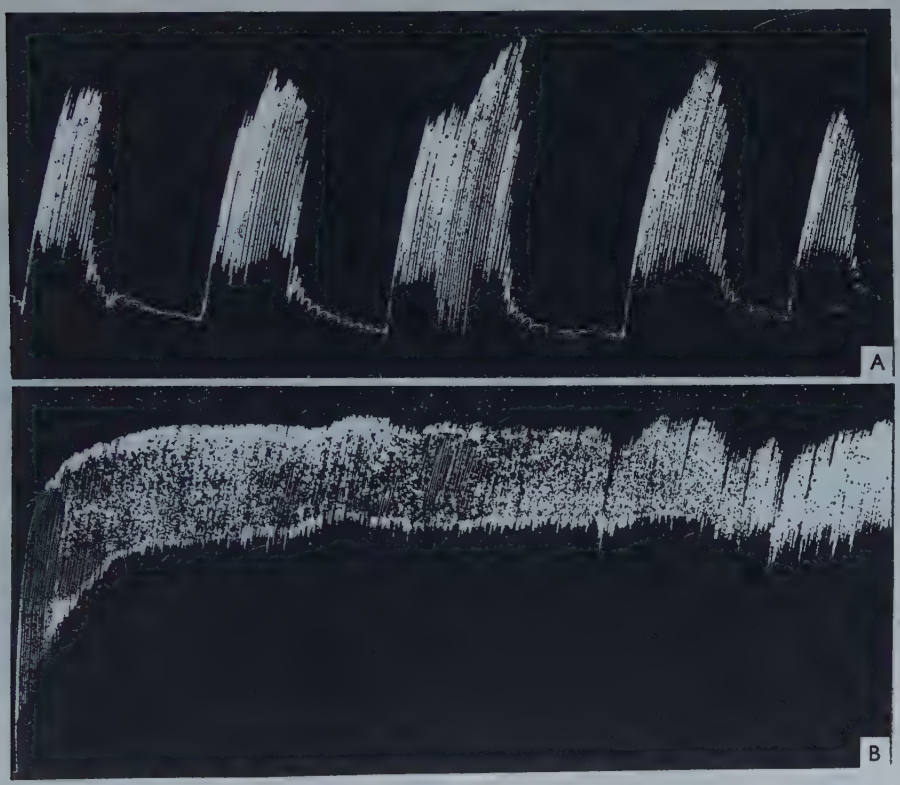

Fig. 4. A: normal activity; B: activity of same specimen after the injection of 6 ml. morphine hydrochloride into foot. Both recordings cover 56 hr. activity.

This is confirmed by the following observations. Injection into the foot of an intact specimen of 5–7 ml. of 2% morphine hydrochloride (which prevents or abolishes the state of tonus in the tonic muscle—see later) allows the rapid rhythm to continue but abolishes the slow rhythm, i.e. the active phase of rhythmical activity persists indefinitely (Fig. 4A, B). Cutting the tonic portions of the adductor muscles has the same effect on the rhythms. These results demonstrate that the slow rhythm is, in fact, a function of the tonic portion, and that the rapid rhythm is exhibited by the phasic portions, but they do not rule out the possibility that the tonic portions may also take part in the rapid rhythm. The only way to settle this point would be to separate the two portions of the muscles and remove the phasic portions completely (merely cutting the phasic fibres from their attachment to the shell is inadequate since rhythmical tensions produced by the continued contractions

of these fibres are transmitted to the valves via the tonic fibres to which they are intimately bound by connective tissue), but this is found to be impracticable since the nerves to the tonic portions pass through the phasic portions of the muscles, and they would be severed when the muscle portions were separated. So, for the time being, this point will have to remain unsettled.

If the cerebropleural ganglia are excised, or if the cerebrovisceral connectives are cut, the posterior adductor muscle goes into a state of increased tonus, drawing the two valves together, and often so vigorously that the movable valve is snapped in two. (It is for this reason that *A. anatina* was sometimes substituted for *A. cygnea*, the former having thicker valves less likely to break). This state of tonus is maintained indefinitely, until the animal becomes moribund after a week or two. During

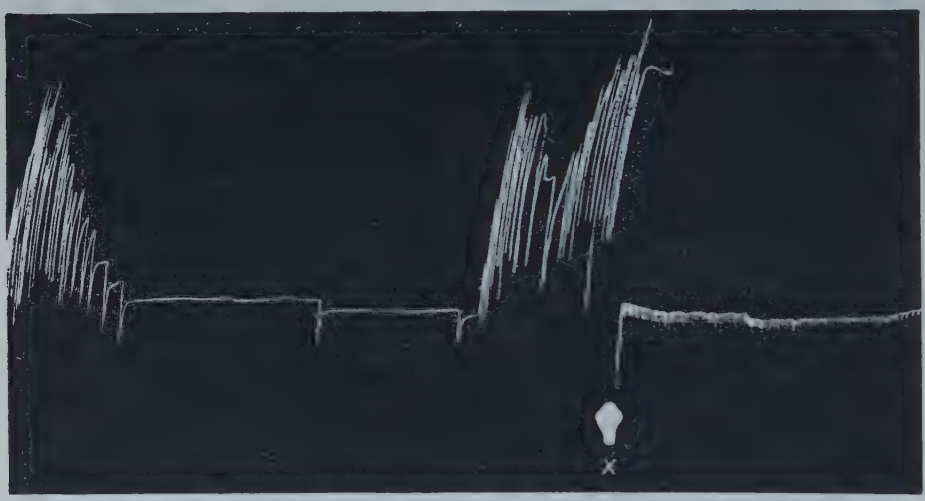

Fig. 5. The effect of 'decerebration' on the activity of the posterior adductor muscle. The first part of the recording is of the activity of an animal intact except for a small window cut in the shell in the region of the cerebropleural ganglia. At *X* the two cerebropleural ganglia were excised, and the anterior adductor muscle was cut (in order to record only posterior adductor activity). Total time, 3 days.

the whole of this time, the phasic portion of the muscle is continuing its rapid rhythmical activity (Fig. 5). The same result is produced if a specimen is completely eviscerated, leaving only the posterior adductor muscle and the visceral ganglia. There are two conceivable explanations of this commencement of tonus: it could be due to the arrest of impulses normally travelling along the connectives, or it could be due to stimulation of the connectives by cutting them or by removing the cerebropleural ganglia. The second explanation is unlikely since electrical stimulation of the connectives has not been observed to induce tonus in the muscle; it has, on the contrary, been repeatedly observed to inhibit a state of tonus. So it may be concluded that the state of tonus is due to the interruption of inhibitory impulses passing along the connectives. Pawlow (1885) demonstrated the presence of inhibitory fibres running from the cerebropleural ganglia to the posterior adductor

muscle, and his conclusions had been confirmed before his paper came to hand. But mere removal of an inhibitory stimulus is not a complete explanation of the assumption by the muscle of a state of tonus; there must be some activating stimulus present in addition. The activating factor might be intrinsic in the muscle fibres themselves, or it might reside in the visceral ganglia. That the visceral ganglia stimulate the muscle to go into a state of tonus is shown by the following experiment. A 'muscle-ganglion' preparation was made, i.e. a preparation consisting of the posterior adductor muscle, the visceral ganglia, an inch or so of the two cerebrovisceral connectives still attached to the visceral ganglia, and sufficient of the movable valve to obtain a recording. The connectives were then subjected to faradic stimulation until the state of tonus in the muscle was inhibited, and the preparation was then left undisturbed. Within 15–20 min. the tonus reappeared, and the process could be repeated. If, however, the ganglia were extirpated while the muscle was in the relaxed condition the tonus did not return. The same observation was made if the ganglia were extirpated in an intact animal during the period of activity when the tonic muscle is naturally relaxed. It therefore follows that as far as the posterior adductor muscle is concerned the slow rhythm is explained by the activity of the visceral ganglia which tend continually to maintain the state of tonus, and of the cerebropleural ganglia which intermittently inhibit that tonus.

The rapid rhythm of the posterior adductor muscle is, on the other hand, independent of the cerebropleural ganglia; as has been said, it is exhibited by a muscle-ganglion preparation. There could be three explanations of this fact: (1) the rhythm could be intrinsic in the striated muscle, as it is, for example, in vertebrate cardiac muscle, or (2) the rhythm might result from proprioceptive reflexes between the muscle and the visceral ganglia, or (3) the rhythm could be intrinsic in the ganglia. Which of these explanations is the correct one has not been satisfactorily settled.

When the visceral ganglia are extirpated from such a preparation the rhythmical activity of the muscle normally stops immediately, but in one specimen of *A. anatina* the rhythm was found to persist for several hours after all visible (under low-power binocular microscope) traces of the ganglia had been removed. (Note: two specimens of *A. cygnea* showed similar continued activity of the anterior adductor muscle after both cerebropleural ganglia had been excised.) Pawlow (1885) recorded a similar observation, but suggested that the continued activity of the muscle was due either to incomplete removal of the ganglia or to the presence of nerve cells in the substance of the muscle. These are probably the most likely reasons for continued rhythmical activity of the muscle, but, nevertheless, the possibility cannot be ruled out that there may be an intrinsic muscular rhythm which normally requires nervous activation for its continuance.

If proprioceptive reflexes are responsible, one would expect that stretching the muscle might modify the frequency of the rhythm, but experiments involving loading the muscle with various weights have yielded inconclusive results. Inducing extra contractions by direct electrical stimulation of the muscle does not

interfere with the rhythm: subsequent spontaneous contractions occur at the approximate times at which they would be expected if no artificially induced contractions had taken place. Furthermore, when the rapid rhythm is of relatively low frequency, recordings often indicate that the muscle is in a state of complete relaxation for several minutes before a spontaneous contraction occurs. These facts suggest that proprioceptive reflexes are not involved in the maintenance of the rapid rhythm.

This leaves the third of the above explanations, namely that the rhythm is intrinsic in the ganglia. Now it has been found that extra contractions of the muscle reflexly induced by electrical or mechanical stimulation of the mantle edge do have the effect of delaying subsequent spontaneous contractions. Since, then, stimulation of the nervous system does affect the rhythm, while direct stimulation of the muscle does not, it seems highly probable that the rhythm is intrinsic in the visceral ganglia. This conclusion must, however, be regarded as only tentative. Probably the only way of confirming it with any certainty is to record the electrical activity of the ganglia before and after their excision, and it is hoped to try this approach to the problem in the near future.

The nervous control of the rhythms in the anterior adductor muscle has not been investigated in the same detail, but the experiments which have been performed suggest a mechanism analogous to that found at the posterior end but entirely under the control of the cerebropleural ganglia.

That these ganglia control the rapid rhythm of the muscle can be demonstrated by injecting a specimen with morphine hydrochloride to cause relaxation of the tonic fibres, cutting across the movable valve so as to record the activity of the anterior muscle only, and then cutting the cerebrovisceral and the cerebropedal connectives and the pallial nerves, leaving only the cerebropleural ganglia connected with the muscle. Such a preparation continues to exhibit the rapid rhythm. If one or other of the cerebropleural ganglia is then extirpated the rhythm still persists, but if both are removed it normally ceases immediately (but see note on p. 165).

The cerebropleural ganglia can be shown also to control the slow rhythm in the anterior muscle. The mere excision of the visceral ganglia is sufficient to stimulate the posterior adductor muscle to go into a state of tonus, from which it may not recover for several days. During this period any continuance of the slow rhythm by the anterior adductor muscle will, of course, be masked. This may be overcome by inserting a fine knife between the shell valves in the position of the exhalant siphon and cutting the tonic fibres of the posterior adductor muscle. It then becomes possible to demonstrate that the slow rhythm can be maintained by the anterior adductor muscle under the control of the cerebropleural ganglia. Even if the cerebropleural ganglion on one side is excised, and the cerebropedal connective and the pallial nerve on the other side are cut, leaving only one cerebropleural ganglion connected to the muscle, the slow rhythm still persists for a few days until the animal dies. If both cerebropleural ganglia are removed all rhythmical activity of the anterior adductor muscle normally stops (Fig. 6).

The conclusions to which these experiments lead can be summarized as follows: each adductor muscle consists of two portions, a phasic and a tonic portion; the phasic portions of the two muscles exhibit rapid rhythmical contractions under the control of the nearest ganglia, while the tonic portions exhibit rhythmically alternating periods of contraction and relaxation under the ultimate control of the cerebropleural ganglia. This control is exercised by the rhythmical inhibition of the contraction produced in each muscle by continuous stimulation from its nearest ganglia.

The question then arises whether the slow rhythm is intrinsic in the neuromuscular system or whether it depends upon some peripheral stimulation or other. It is a simple matter to show that the rapid rhythm is intrinsic because a muscle-ganglion preparation can be made at the posterior end of the animal. The anterior

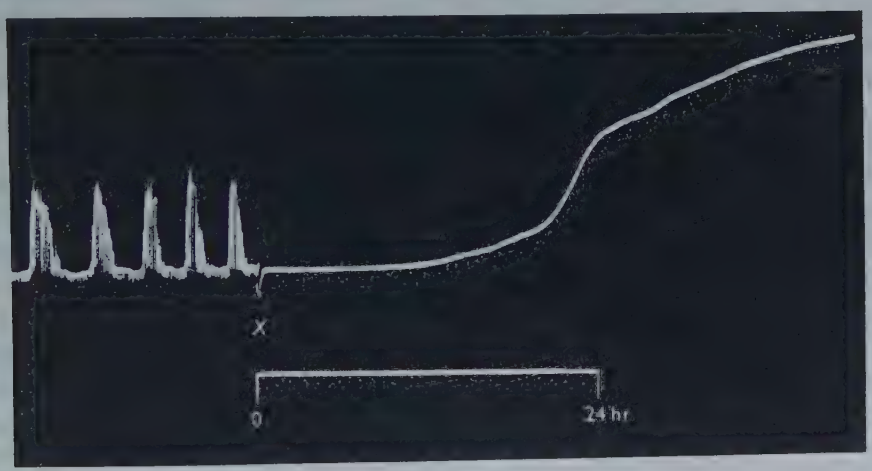

Fig. 6. Activity of anterior adductor muscle under control of the cerebropleural ganglia (visceral ganglia excised, and tonic fibres of posterior adductor cut). At *X* both cerebropleural ganglia were removed.

ganglia and muscle, owing to their anatomical relations, present a much greater problem, and it has so far proved impossible to get a suitable muscle-ganglion preparation. Nevertheless, a tentative answer to the question can be given.

It has been shown that the phase of the slow rhythm which is actively controlled is the period of relaxation of the adductor muscles, and therefore of gaping of the shell valves. If, in fact, peripheral stimulation of the cerebropleural ganglia is responsible, one can guess at the most likely stimuli operating at the end of a period of closure of the valves. They are: (i) lack of oxygen, (ii) excess of carbon dioxide, (iii) accumulation of faeces in the rectum or exhalant siphon, (iv) accumulation of pseudofaeces at some point on the mantle edge, (v) lack of food at some point in the alimentary mechanism, and (vi) accumulation of excreta in the mantle cavity. Experiments have been performed as follows to test whether any of these stimuli are essential for the maintenance of the slow rhythm.

(*a*) Recordings have been made of the activity of an intact specimen in an environment devoid of oxygen. This was achieved by covering the animal with water that had been well boiled and allowed to cool to room temperature in an atmosphere of air that had been passed slowly through alkaline pyrogallol solution. To prevent further oxygen going into solution during the experiment the whole recording apparatus was placed in a sealed glass tank containing a pot of alkaline pyrogallol, and communicating with the outside air only through a wash-bottle containing yet more pyrogallol. The only oxygen then available was that contained within the animal at the beginning of the experiment. These conditions were maintained for 5 days, during which the animal continued to exhibit the usual rhythmical activity.

(*b*) Another specimen was immersed in water through which was passed a continuous stream of carbon dioxide from a Kipp's apparatus. Although the periods of activity were very much reduced in length, the slow rhythm nevertheless persisted for nearly a week. It became somewhat irregular, but this irregularity was shown for a week after fresh aerated water had replaced the CO_2-saturated water, so it must be interpreted as representing permanent damage to the rhythmical mechanism rather than as the result of interference with the normal stimuli.

(*c*) During several different experiments animals were kept under observation for many weeks while immersed in running tap water, so that no food was available from the beginning of the experiments, and no faeces were passed out of the exhalant siphon after the first few days. The regular rhythmical activity nevertheless persisted unaltered.

(*d*) A small notch was cut into the ventral edge of one shell, without damage to the mantle, and a fine glass nozzle was inserted into the mantle cavity, through one of the gills, and thus into the epibranchial space. The nozzle conducted a continuous stream of tap water which washed out the mantle cavity and then escaped via the same notch when the shell was closed and via the exhalant siphon when the shell was open. A flow of 1–1½ l. of water per hour was maintained during the experiment. This, one would imagine, is more than adequate to wash away excreta discharged into the mantle cavity. Normal rhythmical recordings were, nevertheless, obtained.

These experiments between them rule out the possibility that any of the likely stimuli mentioned above are essential factors in maintaining the slow rhythm. Thus experiment (*a*) rules out factor (i), experiment (*b*) factor (ii), experiment (*c*) factors (iii), (iv) and (v), and experiment (*d*) factor (vi). It seems highly probable then that the slow rhythm as well as the rapid rhythm is spontaneous.

THE EFFECT OF MORPHINE HYDROCHLORIDE

Pawlow (1885) had difficulty in performing his experiments on the innervation of the adductor muscles because of the prolonged tonic contractions induced during the dissection of his animals. He therefore decided to narcotize the specimens before dissecting them, and for this purpose he used a 2% morphine hydrochloride solution, which he injected into the foot in sufficient quantity to bring about relaxation of the adductor muscles.

When therefore an anaesthetic was required for a similar purpose in this present work it was decided to use the same drug as the appropriate dosage was already known. But before any operations were performed under the influence of morphine, it was felt desirable to ascertain (*a*) how the drug affected the behaviour of an intact animal, (*b*) how long its effects lasted, and (*c*) whether the normal behaviour returned completely at the end.

For this purpose, 5 or 6 ml. (according to size) of 2% morphine hydrochloride solution were injected into four animals which had been exhibiting normal behaviour for at least a week, and recordings were continued thereafter for 2 or 3 weeks. It was then observed that the injection of the drug had the effect of abolishing the slow rhythm while allowing the rapid one to continue, i.e. the periods of tonic contraction were done away with. This suggested that treatment with morphine might be a useful tool in the investigation of the rhythms, quite apart from its possible use as a narcotic. If it could be discovered at which point in the neuromuscular system it was acting it would indicate a physiological mechanism which is essential in the slow rhythm but inessential in the rapid. This was therefore investigated. The effect upon the posterior adductor muscle of injecting morphine hydrochloride was observed in several specimens which had had various parts of their nervous systems cut or extirpated. In every case the effect was the same—the tonus was abolished. This suggested that the drug influenced the muscle directly and not through the nervous system. This was confirmed by the observation that the injection of 0·5 ml. of the solution into a completely isolated adductor muscle in a state of tonus also led to its relaxation. As Marceau (1909) showed, the tonus is maintained by the unstriated portion only of the adductor muscle, so the site of action of the morphine must be this unstriated portion.

The morphine hydrochloride acts not only upon the adductor muscles. If 5–6 ml. of the 2% solution be injected into an intact animal, it is found that the foot is invariably extended to its fullest degree. This must involve the relaxation of the retractor muscles of the foot as well.

In some experiments it was found that this dose of morphine was sufficient to abolish the tonus of the adductor and retractor muscles, but did not prevent reflex contractions of the adductors and other muscles when various parts of the animals were touched. Slightly increased doses in the same animals, however, did abolish the reflexes, although the rapid rhythmical contractions of the adductor muscles still persisted. Hence, morphine in certain doses does have a narcotic effect upon the nervous system of *Anodonta*; but the observations that Pawlow was making were due not to the narcotic effect, as he supposed, but to the inhibitory effect on the muscle tonus. As a result, some of his conclusions are, in fact, erroneous. Thus he failed to realize that the muscle fibres which he was stimulating reflexly in his morphine-treated specimens are not the ones responsible for keeping the valves adducted during the period of shell closure, and he therefore wrongly identified the tonus of the unstriated fibres with tetanus of the striated ones.

A REFLEX WHICH INHIBITS TONUS OF THE ADDUCTOR MUSCLES

Nadort (1943) described and investigated a number of reflexes exhibited by *A. cygnea*, but, although he searched for one, he failed to find any that led to the opening of the shell by inhibiting the tonus of the adductor muscles.

It seemed to me, however, that in all probability one does exist, since whenever a specimen of *Anodonta* was taken from the storage sink, cleaned, dried, fixed in a dish, and covered with water for the purpose of recording, it invariably commenced to open its shell within a few minutes. The effective stimulus might be one of several: (*a*) the temporary removal from water, (*b*) the agitation of the water as it is run into the dissecting dish, (*c*) the increase in temperature as the specimen is removed from the storage sink and fixed in molten wax in the dissecting dish, (*d*) the decrease in temperature as the new water is run into the dish, or (*e*) the inadvertent shaking and/or rotation of the specimen during its handling.

The following experiment was therefore performed. An animal that exhibited very regular rhythmical behaviour was chosen, and set up intact for recording in the normal way, except that a heavy (and therefore very stable) dissecting dish was used and this was immersed in water in a glass tank sufficiently large to allow the dish to be rotated while still under water. For 9 days a continuous recording was made, and during that time the periods of shell closure were very regular and of approximately 24 hr. duration. It seemed therefore reasonable to assume that the next period of quiescence would be of similar length if the animal were undisturbed. Five hours after it commenced, however, the water surrounding the animal was violently agitated for 2 min., and then left undisturbed for approximately 45 min. No relaxation of the adductor muscles occurred during this time. Then all the water was gently siphoned out of the tank into another container, the specimen was left exposed to the air for half an hour, and the same water, still at room temperature, was once more gently siphoned back. Again no response was observed during the next 1 hr. 40 min. Then the effect of changing the temperature was tried. A lump of ice was suspended in the tank, and the water gently stirred, with the result that a thermometer placed near the specimen indicated a fall of temperature from 15 to 10° C. in 70 sec. The ice was then removed. During the following $\frac{3}{4}$ hr. the shell remained closed. By this time the temperature had risen to 13° C. Some warm water was next poured in, with stirring, and the temperature rose in 10 sec. from 13 to 18° C. The increase in temperature likewise produced no response during the next half an hour. Lastly the animal was disconnected from the recording lever and was, with its dissecting dish all under water, gently rotated 10 times about its longitudinal axis, and then reconnected. The whole process took less than half a minute. Within 5 min. the shell was widely gaping and the rapid rhythm had commenced. This was less than 10 hr. after the last period of activity and therefore 12–14 hr. before the next was otherwise expected. The result of this experiment pointed to the rotation of the animal as being the effective stimulus.

At the time when this conclusion was reached there were seventeen live speci-

mens of *A. cygnea* or *A. anatina* in the storage sink. These were all rotated 10 times without being removed from their water. Before they were rotated only six had gaping shells; 10 min. later all seventeen were gaping.

An attempt was made to analyse the stimulus still further to ascertain whether vibration without rotation was an adequate stimulus or whether the rotation was a necessary part of the stimulating movement. This was done very crudely merely by shaking a number of specimens up and down 10 times in the water of the storage sink without this time rotating them. Whereas seven out of fifteen specimens were

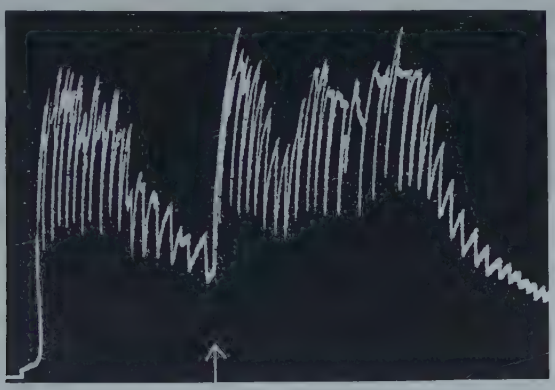

Fig. 7. The effect of vibration on an intact animal. The arrow represents vibration of the laboratory bench due to the placing of a small sink on it. Cf. a normal period of activity of the same specimen (Fig. 2). Total time, nearly 5 hr.

gaping before shaking, twelve were gaping ten minutes afterwards. This result and subsequent observations made at various times during the course of other experiments indicate that vibration without rotation is an effective stimulus, but that specimens vary enormously in their sensitivity to the stimulus. Some require vigorous shaking in the hand, while others respond to vibrations set up by moving a heavy object on the laboratory bench upon which the animal in its dissecting dish is lying (Fig. 7). This latter fact proved to be an occasional source of irregularity in the recordings made of the slow rhythm (see p. 161).

The fact that a much greater proportion of animals responded to vibration with rotation than to vibration alone suggests that rotation is an additional stimulus for the shell-opening reflex. No attempt has been made to identify the receptors for the two stimuli, but it is conceivable that rotation stimulates the statocyst, while vibration causes intermittent pressure of the foot against the mantle lobes. Woortmann (1926) found that the latter was an effective stimulus for shell opening in *Mytilus*, but Nadort (1943) failed to demonstrate this in *Anodonta*; he may, however, have been unfortunate in using specimens of low sensitivity.

DISCUSSION

Lowy (1953) investigated the contraction and relaxation of the adductor muscles of *Mytilus*, and obtained experimental results which he considered favour the 'tetanus' hypothesis of prolonged contraction, although he had difficulty in reconciling certain of his results with this view. The main evidence in support of this hypothesis is that prolonged contraction of the posterior adductor is accompanied by electrical activity of the muscle. Two facts, however, do not accord well with this view, but favour rather the 'catch mechanism' hypothesis of von Uexküll (1929): they are (*a*) the continued state of contraction for about 20 hr. after denervation of the muscle, and (*b*) the occurrence of bursts of muscle potentials during the spontaneous relaxation of the muscle.

If therefore a theory could be propounded which incorporates the 'catch mechanism' explanation of adductor tonus and at the same time offers an explanation of the electrical activity associated with it, it might prove to be the solution to the problem in *Mytilus*. Such a theory is suggested by the foregoing conclusions regarding *Anodonta*.

In *Anodonta* there must be a treble innervation of the adductor muscles, one group of motor nerve fibres supplying the striated muscle fibres and producing phasic contractions (which may summate to produce a tetanus), another group of activating fibres supplying the unstriated muscle fibres and producing increased tonus, and yet a third group of inhibitory fibres supplying the same muscle fibres and producing decreased tonus.

Mytilus, like *Anodonta*, is capable of both phasic and tonic contractions of its adductor, but there is no obvious differentiation of the muscle into two parts, so it must be accepted either that the muscle fibres are all capable of exhibiting both types of contraction or that there are two types of fibres present but completely interspersed. But in either case, the nervous mechanism controlling the adductor activity may be the same as in *Anodonta*. This postulate offers an explanation of apparently all the experimental data in Lowy's paper.

Thus the prolonged tonic contractions such as occur when the mussel is out of water could be regarded as being of the 'catch mechanism' type, and therefore requiring a volley of nerve impulses to bring it to an end. The shorter periods of contraction such as occur when the animal is showing spontaneous activity in sea water could be regarded as periods of tetanus, which would cease when electrical activity ceased. This type of electrical activity would tend to occur in bursts or volleys corresponding to the onset of the contractions, and would be equivalent to the impulses of the rapid rhythm in *Anodonta*. But it has been stated that the rapid rhythm often persists long after a period of tonic contraction has started. If the same thing happens in *Mytilus*, the continued electrical activity during tonic contraction is explained (Lowy has pointed out that this electrical activity is exactly the same as that accompanying spontaneous phasic contractions), as is also its cessation when the visceral ganglia are removed. This hypothesis explains also the continued tonic contraction of the 'deafferentated' posterior muscle-ganglion

preparation, since, if *Mytilus* is like *Anodonta* in this respect, the inhibitory nerves to the posterior adductor muscle arise in the cerebropleural ganglia and merely pass through the visceral ganglia.

Other facts in Lowy's paper could be interpreted in terms of this hypothesis, but enough has been said to show that this hypothesis is worth investigating as a possibly more satisfactory explanation of the neuromuscular physiology of *Mytilus*.

SUMMARY

1. *Anodonta cygnea* L. in captivity exhibits rhythmical behaviour as follows:

(*a*) Periods of activity and quiescence alternate, with frequencies varying with different specimens from 3 to 30 per week. During the periods of quiescence the shell valves are completely adducted. During active periods the valves normally gape. (The 'slow' rhythm.)

(*b*) During the active periods the adductor muscles show frequent rapid contractions followed by slow relaxations. This activity is also rhythmical, and has a frequency of up to 20 per hour. (The 'rapid' rhythm.)

2. The neuromuscular mechanism of these rhythms has been investigated, and it is shown that: (*a*) both adductor muscles participate in the rhythms; (*b*) the slow rhythm is a function of the unstriated portions of the muscles; (*c*) the rapid rhythm is a function of the striated portions of the muscles; (*d*) the rapid rhythm in each adductor muscle is controlled by its nearest ganglia; (*e*) the slow rhythm in each adductor muscle is controlled by the combined effect of the nearest ganglia, which tend always to produce a tonus, and the cerebropleural ganglia, which at intervals inhibit that tonus; (*f*) the control of the rhythms appears to be intrinsic in the nerve ganglia, and independent of peripheral stimulation.

3. The effect of morphine hydrochloride on *Anodonta* has been investigated, and it is shown that, in addition to a possible narcotic effect, it abolishes the tonus of the unstriated portions of the adductor muscles.

4. A reflex is described which leads to the relaxation of the tonus in the adductor muscles, and the commencement of a new period of activity. The stimulus is vibration and/or rotation.

5. On the basis of the above conclusions regarding *Anodonta* a hypothesis is put forward to account for certain facts about the contraction and relaxation of the adductor muscles of *Mytilus*.

I am greatly indebted to Prof. Alastair Graham both for suggesting this investigation, and for providing me with research facilities in the Department of Zoology, Birkbeck College, during a lengthy period when a research laboratory at Chelsea Polytechnic was out of use. He has also kindly read and criticized this paper.

I am also very grateful to Mr C. C. Hentschel, Head of the Department of Botany and Zoology, Chelsea Polytechnic, for research facilities in his department, and to Dr M. F. Lockett, Head of the Department of Physiology, Chelsea Polytechnic, for the loan of physiological apparatus.

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ELECTROPHORETIC MOBILITY OF RED CELLS AND THEIR GHOSTS AS OBSERVED WITH IMPROVED APPARATUS*

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This paper is concerned with the application of two new techniques, both of which depend essentially on improved instrumentation, to the study of the electrophoretic mobility of red cells and their ghosts. The first technique has been developed to overcome difficulties which are encountered in the measurement of the mobility of ghosts. If the ghosts are substantially haemoglobinized, they can be seen, although with some difficulty, with the ordinary microscope and their mobility can be measured in an electrophoresis cell such as that described by Abramson (1934); if they are relatively Hb-free, on the other hand, they are almost invisible under the ordinary microscope, and can be seen satisfactorily only with phase contrast. The great majority of existing measurements of the mobility of ghosts, accordingly, are measurements of the mobility of ghosts which contain relatively large amounts of Hb. The new vertical cell to be described uses phase-contrast optics, and is fitted with removable electrodes which have several advantages over those usually employed. The second technique is the result of the observation that the Antweiler electrophoresis apparatus with a Philpot-Svensson attachment, although designed for the separation and observation of the mobilities of protein fractions, can also be used for measuring the relative mobilities of red cells and their ghosts.

The two principal conclusions reached in the investigations with the ordinary microscope and the Abramson horizontal electrophoresis cell (Abramson, Furchgott & Ponder, 1939; Furchgott & Ponder, 1941) were that the isoelectric point of the haemolysed red cell is in the neighbourhood of pH 2·0, and that ghosts, regardless of the way in which they are prepared (with the exception of the ghost prepared by lysis in CO_2-saturated hypotonic saline), have the same mobility as that of the red cells from which they are prepared. The first conclusion can be confirmed by the improved methods used here, but the second is an over-simplification.

(1) A NEW VERTICAL ELECTROPHORESIS CELL

The construction of the new cell (Fig. 1) is based on that of a vertical cell already described (Ponder, 1951). It differs from the latter in that it has removable electrodes and that the objects (red cells and ghosts) are seen by phase contrast.

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The flat rectangular cell C and the tubes which lead to it are held rigidly on a square frame F of glass rod. Two 3-way stopcocks S_1 and S_2 lead to the female parts of the ground joints of the electrodes E_1 and E_2, into which the removable

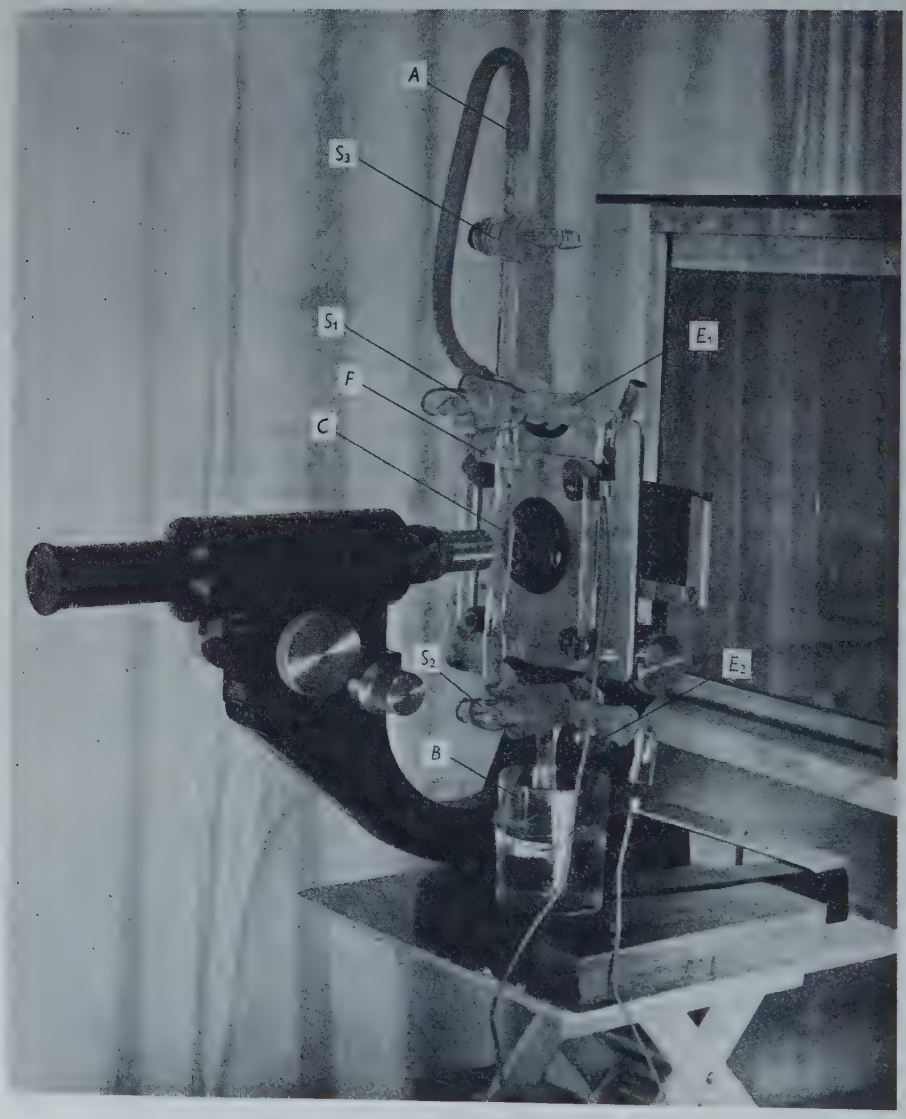

Fig. 1. The vertical electrophoresis cell. The very large water jacket is seen at the right. For description, see text.

non-polarizable electrodes fit as male parts. Each electrode has a short horizontal part and a short vertical part. The end of the horizontal part which fits into the ground joint is closed with a plate of fritted glass about 1 mm. thick, into which some plaster of Paris is rubbed. The end of the vertical part is closed with a snugly

fitting rubber stopper through which a short rod of copper passes. A piece of fine insulated wire, ending in a lug, attaches the copper rod to one of the two terminals of the electrical circuit; by detaching the lug from the terminal, the electrode can be detached from the electrical circuit, and it can also be detached from the electrophoresis cell at the ground joint. The cell is filled by suction through a rubber tube attached at A and empties at B. A 2-way stopcock S_3 has been introduced above S_1; when S_3 is closed, the column of fluid below it behaves as if it were a closed column, almost regardless of the positions of S_1 and S_2.

The frame F is mounted on a metal plate about 1 mm. thick, with a large central aperture through which a long focus phase condenser can project. The plate is clamped, in such a way that it can be removed and returned to exactly the same position, to the square stage of a microscope which is arranged with its stage vertical and its body tube horizontal. The stage and the substage of the standard microscope have to be altered in order that the long-focus phase condenser can be brought close enough to the back of the electrophoresis cell.* The optical system consists of an 8 mm. phase objective ($\times$ 21), a long-focus phase condenser (Bausch and Lomb), and a 25$\times$ eyepiece which carries an eyepiece micrometer disk ruled in squares, each covering about 100μ^2. The light source is a 100 W. projection lamp situated several feet from the electrophoresis cell. A large water jacket and a green filter are inserted in the light path. The electrical circuit consists of a few 45 V. dry batteries, a reversing switch, a milliammeter, and a resistance in series with the cell. The resistance of the cell, when filled with 1% NaCl, is about $15 \times 10^3\ \Omega$.

The cell, which should be cleaned with soapy water followed by many rinses of distilled water, is filled in the following way. The electrodes are first prepared by filling them with saturated $CuSO_4$, inserting the rubber stoppers with their copper rods, rinsing their outsides with water, and applying a thin layer of stopcock grease to their ground surfaces. The cell, on its frame which is permanently attached to the plate, is removed by unclamping the plate from the microscope stage. The rubber tube leading from A is attached to a Mariotte bottle containing saline or buffer. With the stopcocks S_1 and S_2 in position 1 (see inset of Fig. 2), the stopcock S_3 is opened. This fills the female part of the upper electrode; S_3 is closed, and the male part of the upper electrode is inserted into the upper ground joint. Next S_1 and S_2 are turned into position 2; S_3 is opened; the cell and the female part of the lower electrode then fill with saline or buffer. S_3 is again closed, and the male part of the lower electrode is inserted into the lower ground joint. S_2 is now turned into position 3; S_3 is opened, and the tube below S_2 is filled. S_3 is closed again, and S_1 and S_2 are turned into position 4. This procedure fills the cell and connects it with the electrodes. There must be no air bubbles.

* The glass electrophoresis cell was made by E. Machlett and Co. of New York City. The metal plate to which it is attached with its positioning pins and screws, together with the other changes necessary to convert the standard microscope for use with phase contrast are the work of Mr Paul Cutajar of the New York University Machine Shop. The Antweiler apparatus is supplied by Ivan Sorvall of New York City, and was purchased with funds provided by the Eli Lilly Co., Special Grants Committee.

The cell is returned to the microscope stage, to which it is clamped. To fill the cell with a suspension of red cells or of ghosts, it is first emptied by turning S_1 and S_2 into position 3 and opening S_3. The suspension can then be drawn up, from a vessel placed below B, by applying suction to the rubber tube attached to A. The suspension should be drawn up above S_3, which is then closed. S_1 and S_2 are turned into position 4. After a few minutes, during which currents in the fluid become negligible, the cell is ready for the making of mobility measurements at the stationary levels. The distance over which a cell or ghost moves during a short period of time (usually 10 sec.) is observed, first with the current flowing in one direction and then in the other, and the small movement due to gravity or to small systematic drifts (liable to occur in vertical cells) is added or subtracted. The time is much more conveniently measured by the beats of a metronome than with a stopwatch. If large systematic drifts develop, the cell should be emptied and refilled; this takes less than a minute to do. After some hours of work, drifts attributable to diffusion, etc., in the neighbourhood of the electrodes develop; the electrodes should therefore be freshly prepared before the cell is filled at the beginning of each day's work.

Attention should be called to several points. (1) The electrophoresis cell, including the side-tubes which lead to the electrodes, must be filled with the medium in which the cells are suspended. This may be saline or a variety of isotonic buffers, but when the medium is changed the entire filling operation must be carried out anew. If the side-tubes leading to the electrodes are filled with saline, for example, while the cell and the vertical tubes leading to it are filled with a suspension of red cells or ghosts in buffer, the system will soon become unstable and the suspension will begin to stream. (2) Care must be taken not to introduce small air bubbles into suspensions of cells or ghosts, as is easily done when centrifuged material is resuspended by shaking. The upward movement of these small bubbles or of ghosts to which they have stuck is a serious source of instability in the vertical cell. (3) When making measurements of the mobility of ghosts, which are inconspicuous objects even when seen with phase contrast, special care should be taken to be sure that the ghost is in one of the stationary levels. Ghosts tend to move out of these levels more than red cells do, and the best measurements of the mobility of ghosts are those in which the ghost is seen edgewise; when seen edgewise, it almost certainly lies at the stationary level upon which the objective is focused.

(2) A MOVING BOUNDARY METHOD USING THE ANTWEILER ELECTROPHORESIS APPARATUS

The Antweiler microelectrophoresis apparatus (Antweiler, 1952) is essentially a Tiselius apparatus with a Philpot-Svensson attachment, but capable of giving electrophoretic patterns of the proteins in serum, etc., with very small quantities (0·1 ml.) of material and very rapidly (within 10 min.). The light beam which passes through an observation channel is deflected in proportion to the refractive index and therefore to the concentration of the protein in the fluid in the channel, and as the components move under an applied e.m.f., a pattern develops which can

be seen with the Philpot-Svensson attachment. If the channel is filled with a suspension of red cells or their ghosts, these may also move when an e.m.f. is applied, and the position of the boundary between the moving objects, e.g. red cells, and the surrounding medium can be observed with the Philpot-Svensson attachment used without its cylindrical lens. Instead, the channel is observed directly, and the rate of movement, under an applied e.m.f., of the advancing front of the columns of red cells, etc., can either be photographed or measured with an eyepiece micrometer.

Success with this moving boundary method depends on a number of factors. (1) The glass electrophoresis cell which contains the observation channel and the red cells in it must be cooled to between 10 and 15° C. (2) The inclusion of even the smallest air bubble must be avoided. (3) It is essential that the entire system of channels leading to the electrodes is filled with the same fluid as that in which the cells are suspended. This may be isotonic saline or an isotonic buffer. The resistance of the electrophoresis cell obviously depends on the ionic strength of this fluid, but the apparatus is fitted with a voltmeter and a milliammeter, so the field strength can always be determined. (4) When an eyepiece micrometer (conveniently divided into squares with 2 mm. sides) is used to measure the movement of the boundary, the time taken for the boundary to move from right to left over a distance of two eyepiece micrometer divisions determines the mobility, but the initial position of the boundary, before the e.m.f. is applied, ought to be a little to the right of the beginning of the first micrometer division. The reason for this is that the slipping of the upper part of the glass electrophoresis cell over the middle part does not always result in a sufficiently sharp boundary, and it is preferable to allow the boundary between the cells and the surrounding medium to move for half a minute or so before the measurement of the rate of movement is begun. (5) All other conditions being the same, the rate of movement of the boundary is a function of the volume concentration ρ occupied by the red cells or ghosts. The relation is

$$t = t_0 + a\rho,$$

where t is the time required for the boundary to move through a fixed distance (conveniently two micrometer scale divisions), t_0 the time, obtained by extrapolation, for it to move through the same distance if ρ were zero, and a a constant. The constant a varies from one electrophoresis cell to another and must be determined; its numerical value is usually about 4·0. (6) The most reliable observations are made in systems containing red cells or ghosts in a volume concentration of about 0·2. At smaller volume concentrations, the boundary tends to become diffuse.

The necessity for knowing the volume concentration of each suspension of ghosts, so that the rate of movement of the boundary can be compared with that of a red cell suspension of the same volume concentration, is a real objection to the method. In the case of suspensions of some kinds of ghost, e.g. those produced by saponin, the volume concentration is very difficult to determine by centrifuging, although determinations by conductivity methods are still possible; a less specific,

although still important objection is, however, that the electrophoresis cell has to be cleaned and dried before each determination is made. This is very time-consuming.

(3) RESULTS

These are shown in Table 1 and Fig. 2. Apart from showing a general agreement between the relative mobilities obtained with the new vertical cell and with the moving boundary method, the values in Table 1, which are the ratios of the mobility of the object under consideration to the mobility of fresh human red cells under comparable conditions of voltage drop, pH, volume concentration, etc., show that:

(*a*) The mobility of red cells which have been stored at 4° C. for long periods of time is less than that of fresh red cells.

(*b*) Fresh watery ghosts have a considerably higher mobility than the red cells from which they are prepared, but the difference decreases with the length of time during which the ghosts are stored at 4° C.

(*c*) The fragmentation of washed human red cells by heat (53° C. for 5 min.) gives fragments which have a higher mobility than that of unheated red cells.

(*d*) Ghosts made by freezing and thawing or by haemolysis by saponin have substantially the same mobility as the red cells from which they are prepared; this is the conclusion reached by Abramson *et al.* (1939). On the other hand, ghosts prepared by the CO_2 method have a mobility much less than that of the red cells from which they are prepared; this is the conclusion reached by Furchgott & Ponder (1941), except that the reduction in mobility observed here is somewhat larger (a reduction to 0·71 in the red cell mobility instead of the reduction formerly observed, which was 0·82 of the red cell mobility).

These results show that the mobility of ghosts is often different from that of the intact cell. It may sometimes be the same, especially when the ghost is relatively well haemoglobinized, but the mobility is a function of the method by means of which the ghost is prepared and of the time which has elapsed after lysis of the cell. Leaching-out of material from certain types of ghost (Waugh & Schmitt, 1940) may be responsible for the time effects.

There is little doubt that the isoelectric point of the watery ghost is as low as pH 2·0 or even lower. Fig. 2 shows the pH-mobility curve found by Furchgott & Ponder in 1941 and also the curve obtained with the new vertical cell. In the case of the former (dotted in Fig. 2) the pH was controlled by a variety of buffers with ionic strength 0·172, whereas in the case of the latter, Michaelis's universal buffer (acetate and veronal), which has the same ionic strength at all pH's between 2·6 and 9·6, was used. The two pH-mobility curves are not identical but are very similar, and the isoelectric point of the ghost, regardless of whether it is the ghost of a red cell haemolysed at a low pH or a watery ghost suspended in a medium of low pH (as in the case of the observations with the vertical electrophoresis cell) is certainly situated at a pH of 2 or even less.

Table 1

(To convert to mobilities in μ/sec./V./cm. multiply all values by $-1{\cdot}06$.)

Material	Vertical cell	Antweiler apparatus
Fresh human red cells in buffer at pH 8·6	1·00	1·00
Red cells stored for 20 days at 4° C.	0·95	—
Red cells stored for 100 days at 4° C.	0·91	0·89
Fresh watery ghosts	1·30	1·35
Watery ghosts stored 7 days at 4° C.	1·10	1·08
Watery ghosts stored 14 days at 4° C.	1·02	1·00
Fragmented red cells, 53° for 5 min.	1·18	1·17
Fresh ghosts, freezing and thawing	1·00	1·00*
Ghosts prepared in CO_2-hypotonic saline	0·70	0·72
Ghosts prepared with saponin, 1 in 10^3	1·00	—†

* Difficult to measure.
† ρ cannot be determined by haematocrit.

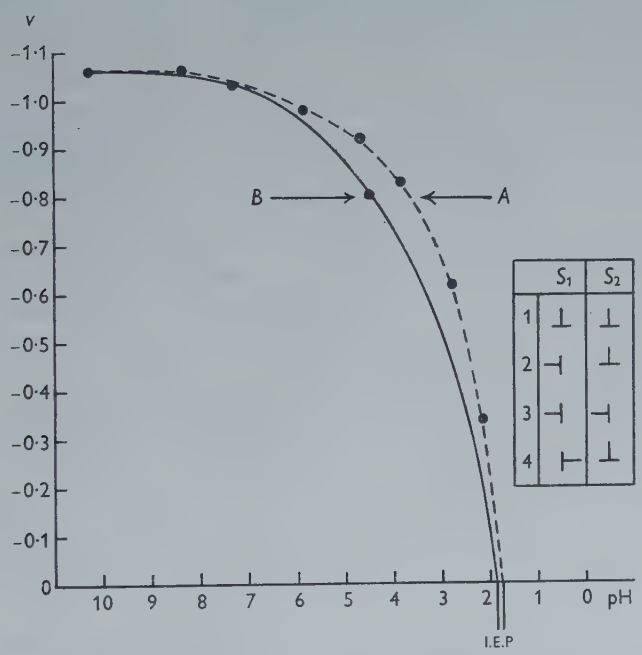

Fig. 2. pH-mobility dependences for human red cell ghosts at constant ionic strength. Ordinate: mobility v in μ/sec./V./cm.; abscissa: pH. I.E.P., isoelectric point. Curve *A* (dotted) is the relation obtained by Furchgott & Ponder in 1941; curve *B* is the relation found with the new electrophoresis cell for systems of red cells and ghosts in Michaelis's universal buffer. Inset shows the positions of the stopcocks S_1 and S_2, during the filling of the cell described in the text.

SUMMARY

Two new techniques for measuring the electrophoretic mobility of red cells and ghosts are described. In the first, the mobilities are measured in a vertical electrophoresis cell in which they are seen by phase contrast. This enables mobilities to be measured even when ghosts contain so little Hb as to be invisible with the

ordinary microscope. The second method uses an Antweiler electrophoresis apparatus to give measurements of the mobility of moving boundaries between columns of red cells or ghosts and the suspension medium.

It can be shown by these methods that the usual statement that the mobilities of ghosts are the same as those of the red cells is an over-simplification. The mobilities, under comparable conditions, are more generally dependent on the method used to prepare the ghosts and on the time which has elapsed after lysis.

The statement that the isoelectric point of watery ghosts is as low as pH 2·0 or even less is confirmed, as is also the general shape of the pH-mobility dependence.

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THE EXCRETORY SYSTEM OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE)

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INTRODUCTION

The work which is to be described in this paper is in a sense a by-product of an investigation which is now being made of the process of urine formation in the Malpighian tubules of insects. The stick insect has been chosen as a suitable animal upon which to begin this investigation largely because of the relative ease with which its tubules can be studied as isolated preparations. Some of this work has already been published (Ramsay, 1953*b*, 1954, 1955). The lack of information upon the more general aspects of excretion in this insect has made itself felt, and for this reason it was decided that a survey of the whole excretory system would have to be undertaken.

Although the Malpighian tubules are commonly regarded as the excretory organs of insects it is becoming increasingly clear that this conception is misleading. Many of the substances which are excreted in the urine—by which is meant the fluid issuing from the tubules—are reabsorbed in other parts of the gut, in particular in the rectum. The excretory system is here understood to be the Malpighian tubules, together with the whole of the hindgut; but even this wider definition may not always be adequate since it does not include regions of the body in which excretory matter may be stored (e.g. uric acid in the fat-body of the cockroach) and takes no account of other routes (e.g. the genital system) by which, as so happens in the stick insect, the bulk of the calcium leaves the body.

It is necessary to correct any impression that all possible technical resources, even the limited technical resources available for physiological studies upon small animals, have been brought to bear upon this problem. Rather, it must be emphasized that this work is in the nature of a preliminary study and aims no further than to establish the broad outlines, leaving much detail still to be filled in.

MATERIAL AND METHODS

Except where otherwise stated, the results reported in this paper refer to the adult female stick insect, bred in the laboratory on the leaves of privet.

The methods used for the collection of fluids from various parts of the body cannot conveniently be summarized here, but will be described briefly in the appropriate context.

The methods of analysis used were as follows. Sodium and potassium; flame photometry (Ramsay, Brown & Falloon, 1953). Calcium; precipitation (twice) as

oxalate and titration with ceric sulphate (Kirk, 1950). Magnesium; titan yellow method (Orange & Rhein, 1951). Chloride; potentiometric titration with silver nitrate using apparatus devised by Mr P. C. Croghan, whom I wish to thank for instruction in the method. Phosphate; ammonium molybdate method as described by Delory (1949), adapted for 1 ml. cells. Uric acid; Benedict's arsenophosphotungstic method as described by Delory, adapted for 1 ml. cells. Osmotic pressure; cryoscopic method (Ramsay, 1949). pH; micro glass electrode (Hartree, 1952); I am indebted to Dr E. W. McConnachie for this measurement. Paper chromatography has been used as a qualitative method.

ANATOMY AND HISTOLOGY

A general account of the Phasmidae has been given by de Sinéty (1901). His description goes into great detail of some regions of the gut and its appendages while others, e.g. the whole of the hindgut, receive no mention at all.

The general arrangement of the excretory system is illustrated diagrammatically in Fig. 1. As is commonly the case in primitive insects the Malpighian tubules arise from an annulus which marks the division between midgut and hindgut.

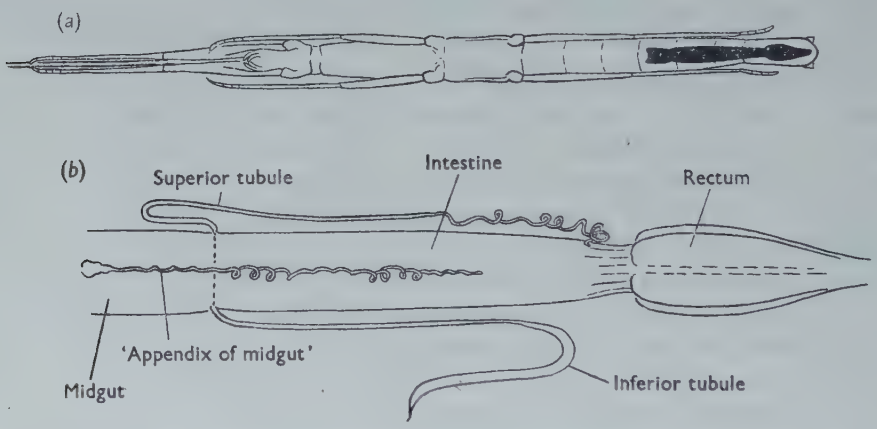

Fig. 1. (*a*) To show position of region illustrated in (*b*). (*b*) Malpighian tubules and posterior region of gut.

There is no sphincter or constriction in this region. The anterior region of the hindgut, which will be called the intestine, is separated from the short rectum by a sphincter which is normally closed. The three types of Malpighian tubule recognized by de Sinéty are distinguished most readily by their anatomical relations with the gut and other structures.

(*a*) The superior tubules arise from the annulus, run forward a short distance and then turn back following the intestine to which each tubule is attached by several short tracheae. These arise from the tracheal supply which invests the hindgut and on reaching the tubule each trachea divides into ascending and descending branches which supply a short length of tubule. Towards the distal

(blind) end the tubule is more contorted and ends at about the level of the rectal sphincter. The tubules of the nymph correspond to the superior tubules of the adult.

(*b*) The inferior tubules arise from the annulus in pairs having a very short common trunk and run at first directly backwards. The distal region of the inferior tubule, which may represent one-third or more of its total length, is in the form of a dilatation containing white granules; it is not closely applied to the gut but lies free in the posterior body cavity being attached at its blind end by short tracheal branches to the connective tissue of the fat-body. The blind end itself is provided with a cap of cells of vesicular appearance, the cells of Sirodot, between which the tracheal branches pass to spread out over the distal dilatation. The only other tracheal supply is a single branch which applies itself to the tubule at the proximal end and runs in a loose spiral over the proximal and middle regions. There are no inferior tubules in the nymph and they are poorly developed in the male (de Sinéty).

(*c*) The tubules of the third kind ('appendices of the midgut') open separately into small pyriform dilatations of the midgut wall anterior to the annulus. They are thinner than the superior and inferior tubules and more contorted. They run directly back to end close to the intestine about one-third of the distance from the annulus to the sphincter.

All three types of tubule are provided with muscular elements running in loose spirals over their walls and can be seen to undergo gentle writhing movements.

In each of two insects an accurate count was made of the tubules of the three types, which were found to be present in the following numbers: superior tubules 24, 23; inferior tubules 134, 134; appendices of the midgut 32, 25.

The appearance of the epithelium in the different regions of the gut has been studied in sections fixed in Susa, cut at 10μ and stained in iron haematoxylin. At the region of the annulus the columnar epithelium of the midgut gives way to the cubical epithelium of the intestine and this in turn to the tall columnar epithelium of the six rectal glands. These epithelia are illustrated in Fig. 2.

The Malpighian tubules have been examined alive in haemolymph and are illustrated in Fig. 3.

(*a*) *Superior tubules*. When observed under the low power the cells of the healthy tubule appear as a bright transparent colourless wall whose inner margin is made discernible by the presence of granules of various kinds and in varying amount. In the middle and proximal regions the granules have a greenish yellow colour, in the distal region they are white and the tip is generally devoid of granules. When the insect has been feeding regularly very few granules are present, but when the insect has fasted for 3 or 4 days the tubule appears to be packed with granules which, however, are not free in the lumen but are attached in masses to the inner margin of the wall. These granules are soluble in dilute alkali but not in dilute acid. An alkaline extract of the tubules of fasting insects contains more than 20 times as much uric acid as a similar extract of the tubules of fed insects. This suggests that these granules contain uric acid; they do not, however, have the appearance of the

uratic spheres known from the urine of *Rhodnius* nor do they appear to be enmeshed by the filaments of the brush border as in that insect (Wigglesworth, 1931*a*, *b*).

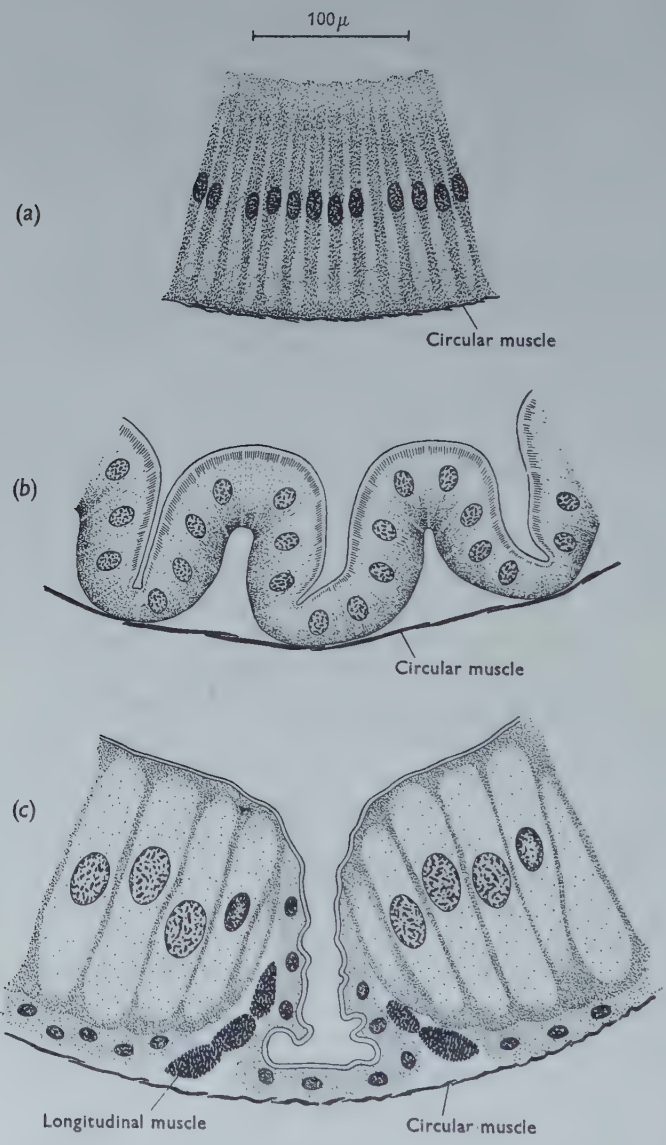

Fig. 2. Wall of gut in transverse section. From material fixed in Susa and stained in iron haematoxylin. (*a*) Midgut. (*b*) Intestine. (*c*) Rectum.

Over most of the length of the tubule the cells (which are bi-nucleate, de Sinéty) are provided with a well-developed brush border which has no clearly defined inner margin and is therefore of the 'bürstensaum' type (Wigglesworth, 1931*b*). At the proximal end the diameter is greatest, about 120μ, and the brush border is

deep, but with very fine striations and a very indefinite inner margin. Towards the middle region the diameter decreases to about 100μ, the brush border becomes a little less deep and the striations (filaments) are clearer and all of the same length which defines the inner margin, although no surface can be brought to focus (Fig. 3*b*). The diameter of the distal region is about 80μ. Over the last 2 or 3 mm. at the tip the character of the border changes. The filaments are shorter and gathered into clumps, rather in the way that the hairs of an animal's fur are held together by surface tension when wet, and the inner margin can be brought to focus (Fig. 3*a*).

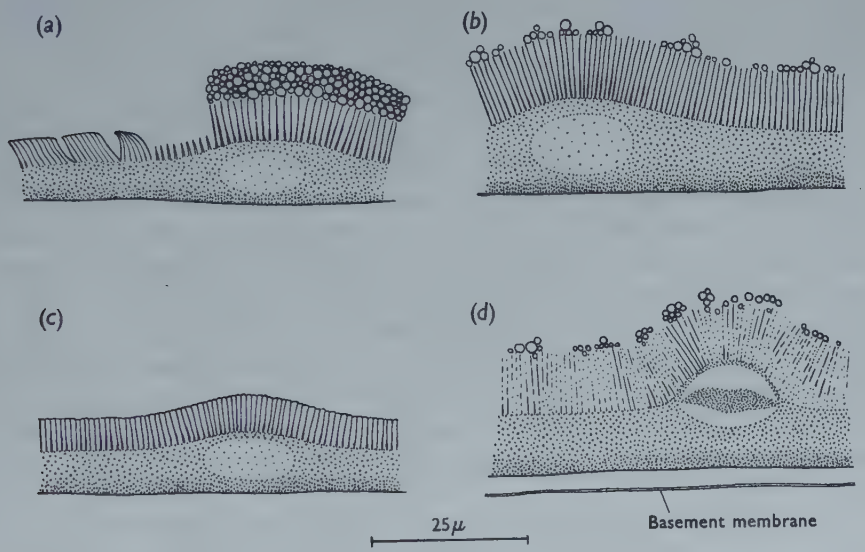

Fig. 3. Walls of Malpighian tubules. From living material. (*a*) Superior tubule, distal region, showing change in brush border near the tip (left); in serum. (*b*) Superior tubule, middle region; in serum. (*c*) 'Appendix of midgut'; in serum. (*d*) Superior tubule, middle region, after 2 hr. in Ringer solution, showing changes in nucleus and brush border.

(*b*) *Inferior tubules.* Over their middle and proximal regions the inferior tubules present the same appearance as the superior tubules, apart from the fact that they contain rather fewer granules. The distal dilatations, 250–300μ in diameter, are provided with a brush border of what appears to be the same type as found in the proximal and middle regions; this can be established in favourable cases where the dilatation is not completely packed with granules. The cells of Sirodot have not been studied further.

The contents of the distal dilatation were analysed qualitatively in the related phasmid *Leptynia attenuata* by de Sinéty. He demonstrated the presence of carbonate by microdiffusion of CO_2 into baryta. He claimed to have identified uric acid, oxalate and leucine upon crystal form but without any more specific tests. In the course of the present work carbonate has been confirmed but not oxalate or leucine. Uric acid is present in small amounts. There is a great deal of calcium and only a trace of magnesium. It is concluded that the principal constituent of

these granules is calcium carbonate. The egg shells of phasmids are hardened with calcium oxalate (Moscona, 1950). Since the inferior tubules with their dilatations and granules are absent in the nymph and poorly developed in the male, and since the granules appear to be reduced in quantity during fasting (egg production continuing), it seems likely that they are a calcium reserve which can be drawn upon to provide material for the egg shells.

(*c*) *Appendices of the midgut.* These tubules are narrower (*c.* 50μ) than the others and are uniform throughout their length. There is a well-developed brush border and the inner margin can be brought to focus (Fig. 3*c*). It is perhaps less sharply defined than the scalloped edge of the 'wabensaum' in *Rhodnius* (Wigglesworth, 1931*b*), but more definite than the edge of the border as seen at the tip of the superior tubule (Fig. 3*a*). No granules have been seen in these tubules.

Wigglesworth has reported that in *Rhodnius* the normal histological appearance of the tubules is not preserved in artificial media. The same is true of the tubules of the stick insect in all artificial media as yet tested. A Ringer solution approximating to haemolymph in composition has been prepared (Ramsay, 1955). Within an hour or two after being placed in Ringer the walls of the tubules begin to lose their characteristic bright appearance. The striations of the brush border become fainter and the border eventually disintegrates. The appearance of the nuclei changes with what appears to be the separation of a coagulum from a clear fluid within the nuclear membrane (Fig. 3*d*). A space appears between the cells and the basement membrane which is clearly visible only under these conditions, and after 5 or 6 hr. a disintegrating mass of cells fills the lumen. The contraction of the muscular elements in the walls continues for some time longer.

Table 1. *Relative quantities of mineral bases as percentage of total mineral base expressed in equivalents*

	Privet leaves (1)	Nymph faeces (2)	Adult faeces (3)	Eggs (4)
Na	13	9	11	10
K	35	33	66	5
Ca	46	51	8	83
Mg	6	7	15	2
	100	100	100	100

EXCHANGE OF MINERAL BASES

The insect takes in sodium, potassium, calcium and magnesium in the proportions in which they are present in privet leaves (Table 1, col. 1), and should presumably eliminate them in the same proportions. In the case of the nymph elimination is entirely by way of the faeces, and it is found that in the faeces of the nymph the four bases are present in much the same proportions as in the leaves (Table 1, col. 2). In the case of the adult female the eggs constitute a second channel of elimination. The rate of egg production is considerable and in terms of ash a somewhat greater weight is eliminated as eggs than as faeces. It is difficult, however, to

make out a balance sheet since there is at any time a large number of eggs in the body at various stages of development, and a large and variable store of calcium in the inferior tubules. Cols. 3 and 4, Table 1, show that most of the potassium and magnesium is eliminated in the faeces while most of the calcium is eliminated in the eggs.

THE HAEMOLYMPH

No extensive analysis of stick-insect haemolymph, comparable with Levenbook's (1950) analysis for *Gastrophilus*, has yet been made, but a certain amount of information from various sources is assembled in Table 2, col. 1. The stick insect conforms to the usual pattern of the herbivorous insect in that its haemolymph contains more potassium than sodium and more magnesium than calcium. It also contains a great deal of phosphate and appears to be supersaturated with respect to magnesium phosphate.

Table 2

	Haemolymph (1)	Serum (2)	Urine (3)
pH	—	6·6	6·8–7·5
Δ	160 mM./l. NaCl*	171 mM./l. NaCl	171 mM./l. NaCl
Na	8·7 m.equiv./l.†	11 m.equiv./l.	5 m.equiv./l.
K	27·5 m.equiv./l.†	18 m.equiv./l.	145 m.equiv./l.
Ca	16·2 m.equiv./l.†	7 m.equiv./l.	2 m.equiv./l.
Mg	145 m.equiv./l.†	108 m.equiv./l.	18 m.equiv./l.
Cl	93 m.equiv./l.‡	87 m.equiv./l.	65 m.equiv./l.
PO_4^{3-}	120 m.equiv./l.	39 m.equiv./l.	51 m.equiv./l.
Uric acid	10·4 mg./100 ml.§	4·5 mg./100 ml.	43 mg./100 ml.

* Rouschal (1940). † Duchâteau *et al.* (1953). ‡ May (1935). § Florkin (1936).

The fresh haemolymph of the stick insect is not a very convenient medium for physiological work since it almost invariably coagulates during the course of an experiment. It has been found that if the fresh haemolymph is heated to 100° C. for about 5 min. and then centrifuged, a fluid, which will here be called serum, is separated from a compacted clot. This fluid still preserves the characteristic blue-green colour of fresh haemolymph which, according to Abeloos & Toumanoff (1926) is due to 'carotinalbumines'. As a physiological medium for Malpighian tubules it does not appear to be in any way inferior to fresh haemolymph, and has been extensively used in an investigation which is reported elsewhere (Ramsay, 1955). An analysis of serum is given in Table 2, col. 2. The concentrations of calcium, magnesium and phosphate are reduced as compared with haemolymph, and it is probable that earthy phosphates have been precipitated and removed with the clot. Even if all the phosphate is present as PO_4^{3-}, which is unlikely, there is an anion deficit of about 20 m.equiv./l. in serum.

A word of comment is needed on the subject of sodium and potassium concentrations. The figures given by Duchâteau, Florkin & Leclercq (1953) show a lower concentration of sodium and a higher concentration of potassium than do the figures for serum (Table 2, cf. cols. 1 and 2). This difference is not due to the heat-treatment of serum since the same concentrations are found in the haemolymph

from which serum is prepared. Later in this paper (Table 5) further figures are given for haemolymph, showing higher sodium and lower potassium than in serum. When this inconsistency was discovered investigation was made into its cause. The collections of haemolymph from which serum was prepared were made by cutting off a leg and drawing up the haemolymph into a tube, the exudation of haemolymph being assisted by light pressure applied to the insect's body with the fingers. A number of insects were subjected to this treatment and then dissected, and in some of them it was found that the midgut was perforated near the annulus. The effect of this would be to contaminate the haemolymph with urine having a low sodium and high potassium concentration. It is possible that similar contamination affected the collections made by Duchâteau *et al.* (1953) in greater degree.

Duchâteau, Sarlet & Florkin (1952), using methods of biological assay, have given an extensive list of amino-acids, free or combined in a non-protein form, found in stick-insect haemolymph. In the present work a brief investigation using paper chromatography was made of haemolymph de-proteinized with trichloracetic acid. Only two ninhydrin-positive compounds which withstood hydrolysis with concentrated hydrochloric acid were found in substantial concentration; one of these was glycine, the other could not be identified by R_F values. An imposing array of spots appeared after hydrolysis, but these were not further studied. It is concluded that most of the amino-acids recoverable from de-proteinized haemolymph are present as peptides.

The total volume of the haemolymph in a stick insect can be found approximately in the following way. A leg is cut off, as much haemolymph as possible is collected and its volume measured in a capillary pipette. An equal volume of Ringer solution is then injected, and after about half an hour has been allowed for it to become uniformly distributed a second sample of haemolymph is taken. Both first and second samples are then heat-coagulated and centrifuged. The dilution of the second sample is found by adding Ringer to the first sample until the same depth of blue-green colour is obtained. A simple calculation then gives the total volume of haemolymph in the insect. Figures thus obtained ranged from 76 to 186 mm.^{3} with an average value of 132 mm.^{3} for six insects. The average weight of an adult female stick insect is 0·8 g., so that the haemolymph represents about 15% of the insect's weight.

THE URINE

Sufficient urine to make possible the analyses in Table 2, col. 3, was collected in the following way. The insect was fastened down on its back with plasticine, and the abdomen was opened along the mid-ventral line. A cotton-thread ligature was tied around the intestine just posterior to the annulus, passing between the intestine and the Malpighian tubules so as to leave the latter free. A slit was then made into the midgut wall about 2 mm. anterior to the annulus, the gut contents were removed and a cannula was tied into place, the ligature passing just anterior to the annulus. In this way it was possible to collect the fluid, presumably urine, accumulating in the short stretch of gut between the ligatures. Urine was produced at rates of

4·0–6·5 mm.³/hr. The urine rose in the cannula as a pale yellow fluid soon becoming dark brown on exposure to air.

As was already known, the potassium concentration is higher and the sodium concentration is lower in the urine than in the haemolymph. Calcium, magnesium and chloride are lower in the urine, but phosphate is higher. There is an apparent anion deficit of some 50 m.equiv./l. Uric acid is in higher concentration in the urine as might be expected.

CHANGES IN THE URINE DURING ITS PASSAGE THROUGH INTESTINE AND RECTUM

In this and in the next section consideration is restricted to water, sodium and potassium. Methods of analysis suitable for the small volumes available have not yet been developed for other constituents of the urine.

It is immediately obvious that with urine being produced at a rate of about 6 mm.³/hr. all the water would be removed from the haemolymph in less than 24 hr. if it were not reabsorbed in the hindgut. It is also obvious that all the potassium in the haemolymph would be removed in less than 3 hr. and therefore potassium, like water, must presumably be reabsorbed. To demonstrate such reabsorption is not altogether a straightforward problem since besides the urine semi-digested food containing potassium is being passed into the hindgut at an unknown rate. This complication can be avoided by using fasting insects.

For these experiments the insects were kept without food in glass jars lined with moist filter-paper, in which they could survive for more than 10 days. The production of faeces fell off rapidly from about 20 mg. to about 2 mg. dry weight per insect per day. 20 mg. of 'fasting' faeces were ashed and found to contain 0·3 μequiv. of sodium and 18 μequiv. of potassium; this indicates a loss of 0·03 μequiv. of sodium and 1·8 μequiv. of potassium per insect per day. From figures already quoted it can be calculated that the Malpighian tubules will excrete 0·7 μequiv. of sodium and 21 μequiv. of potassium in one day, so that considerable reabsorption must take place.

This of course presupposes that the rate of flow and composition of the urine are maintained at the same levels in the fasting insect. The conspicuous accumulation of granules in the Malpighian tubules of the fasting insect suggest at first sight that the flow of urine must be reduced almost to zero, but in fact this is not so. Collections of urine made in parallel experiments on fed and fasting insects showed only a small decline in rate of flow, the figures being 5·4 and 6·4 mm.³/hr. for fed insects and 4·2 and 2·9 mm.³/hr. for fasting insects.

Sodium and potassium concentrations in the urine of fasting insects are given in Table 3, col. 3, and when these are compared with the figures in Table 2, col. 3, it can be seen that the sodium concentration is slightly greater and the potassium concentration is slightly less in 'fasting' urine as compared with 'fed' urine. Taking rate of flow as 3·5 mm.³/hr., sodium concentration as 6·5 m.equiv./l. and potassium concentration as 125 m.equiv./l. the quantities excreted by the Malpighian tubules of a fasting insect per day are 0·54 μequiv. of sodium and 10·5 μequiv.

of potassium, so that about 95% of the sodium and about 80% of the potassium must be reabsorbed.

The fact of reabsorption being established, the next question to be asked is whether this takes place exclusively in the hindgut or whether there is a forward movement of urine into the midgut as may occur in the mosquito larva (Ramsay, 1953*a*). This was tested by injecting phenol red into the body cavity. The dye is quickly excreted by the Malpighian tubules, and when the insect is opened it is found to be present in the intestine and rectum. On one occasion only was the dye found in the midgut and then only a few millimetres anterior to the annulus. It is therefore in the intestine and rectum that we may expect to find reabsorption of urinary constituents.

Table 3. *Haemolymph, intestinal fluid and urine from three fasting insects*

Haemolymph (1)			Intestinal fluid (2)			Urine (3)		
Na	K	O.P.	Na	K	O.P.	Na	K	O.P.
15	11	160	7	102	162	5	120	150
11	10	173	5	101	172	7	113	162
15	10	155	6	133	166	7	135	158

O.P. = osmotic pressure.

Table 4. *Rectal fluid from six feeding insects*

Na	K	O.P.	Na	K	O.P.
21	530	551	22	320	449
8	225	255	29	415	520
11	160	199	21	310	364

It is well known from the work of Wigglesworth (1932) that the rectal glands of insects are concerned in the reabsorption of water. It is sometimes possible, by gentle pressure upon the abdomen, to force a drop of fluid out of the stick insect's anus. Analyses of such drops from six insects are given in Table 4. The high values of osmotic pressure indicate the withdrawal of water which is to be expected. The sodium/potassium ratios are very variable, and since these measurements were made on fed insects they are difficult to interpret.

It is also possible that reabsorption takes place in the intestine. Analyses of intestinal fluid from fasting insects are given in Table 3, together with analyses of urine collected in the usual way after the samples of intestinal fluid had been taken. The composition of intestinal fluid is seen to be substantially the same as that of urine, and there is no indication of increased osmotic pressure as in rectal fluid.

The possibility still remains, however, that there is reabsorption of urine in the intestine without substantial change in its composition. This possibility was studied in two ways. According to the first method the insect was opened and

ligatures were tied around the rectal sphincter and around the midgut just anterior to the annulus. After some hours the intestine was seen to be abnormally distended. A third ligature was then tied just posterior to the annulus, and a few hours later the distension of the intestine was noticeably less. According to the second method a cannula was filled with urine from another insect and was tied into the intestine by a ligature just posterior to the annulus, the rectal sphincter being ligatured as before. A small pressure (1·5 cm. of water) applied to the cannula kept the intestine slightly distended. The urine was gradually absorbed at rates of 1·5 and 0·9 mm.^{3}/hr. in the two experiments performed. The urine remaining in the intestine and cannula was analysed after the experiment and was not found to have changed appreciably in composition.

It is therefore concluded that the main site of reabsorption of sodium, potassium and water is the rectum, and that while some reabsorption can take place in the intestine this is probably inconsiderable under normal conditions (but see next section).

THE ROLE OF THE EXCRETORY SYSTEM IN REGULATING THE COMPOSITION OF THE HAEMOLYMPH

The figures presented in the early part of the previous section show that the excretory system is responsible for a rapid turnover of sodium, potassium and water, and that this is not incompatible with a haemolymph of reasonably constant composition. As Boné (1944) first showed, the sodium/potassium ratio in the haemolymph of insects is correlated with the diet, being high in carnivorous and low in herbivorous insects. Boné did not go so far as to suggest that this was a *direct* result of the high potassium intake of herbivorous insects, but a simple relationship of this kind is not excluded as a possibility. Tobias (1948) was able to lower the sodium/potassium ratio in the haemolymph of the cockroach by feeding the insect on lettuce, but not to the level characteristic of truly herbivorous insects. Hoyle (1954) has recently shown that in the locust the potassium concentration in the haemolymph falls by about 50% during starvation; it appears that in the locust the normal composition of the haemolymph represents the balance struck between processes of assimilation and excretion.

It was therefore natural to begin the investigation of this matter in the stick insect by following the changes in the composition of the haemolymph during starvation. Six insects were isolated without food in glass jars lined with moist filter-paper. Samples of haemolymph, about 1 mm.^{3} in volume, were collected by thrusting a pipette through the arthrodial membrane at the base of a leg. The first sample was taken immediately after the insect had been removed from a cage containing privet leaves, the second sample after 48 hr. and the third sample after 96 hr. starvation. The analyses of these samples are given in Table 5. In all cases except one there is a slight ($<20\%$) decrease in the potassium concentration while the sodium concentration and osmotic pressure remain relatively constant. Quite clearly the effect of starvation is much less in the stick insect than in the locust.

The next step was to inject solutions into the haemolymph so as to alter the concentrations and then to follow the process of return to normal. For these experiments the same six insects were used. Immediately after the last sample of haemolymph had been taken (at 96 hr.) the insects were injected, nos. 1–3 with 50 mm.^{3} of 170 mM./l. NaCl and nos. 4–6 with 50 mm.^{3} of 170 mM./l. KCl. After 1 hr. had been allowed for the injected solution to become uniformly distributed about the body the first sample was taken, and subsequent samples at 6, 24, 72 and 96 hr. reckoned from the time of injection. As soon as the last sample had been taken the insect was opened, a sample of intestinal fluid was taken and a small sample of urine was collected in the usual way.

The effects of injection of NaCl and KCl were unmistakeably different. The NaCl-injected insects appeared to be normal in every way, whereas the KCl-injected insects quickly lost the ability to make co-ordinated movements. Of these no. 4 showed a considerable measure of recovery, but nos. 5 and 6 remained completely paralysed for 24 hr. and were judged to be dead, although in the case of no. 6 it seems that eventual recovery may still have been possible. Had this been realized at the time, the attempt would have been made to collect urine from no. 6.

The results of analyses are assembled in Table 6. Assuming that the normal concentration of sodium (and of potassium) is 15 m.equiv./l. and that the volume of the haemolymph is 135 mm.^{3} the injection of 50 mm.^{3} at 170 m.equiv./l. should raise the sodium (or potassium) concentration in the haemolymph to about 55 m.equiv./l. Concentrations of this order, though somewhat lower, are found 1 hr. after injection.

In insects nos. 1–3 the sodium concentration fell slowly towards the normal value, but this change had only gone about half way in 3 days. The sodium concentration in the urine is about 3 times its usual 'fasting' level, while the potassium concentration is not increased. It is to be noted that in all three cases there is a substantial rise in sodium concentration and fall in potassium concentration in the intestinal fluid as compared with the urine.

These three insects continued to produce faeces which were collected and analysed. The sodium content was found to be much greater than in 'fasting' faeces and the potassium content less. The rates of excretion per day worked out at 1·1 μequiv. of sodium and 0·85 μequiv. of potassium; that is to say, in the NaCl-injected insect the rate of sodium excretion is more than 30 times greater and the rate of potassium excretion is more than 50% less than in the fasting insect.

There is therefore clear evidence of an adaptive response by the excretory system to an increase of the sodium concentration in the haemolymph.

On the other hand, the adaptive response is not a very effective one when seen in relation to its corrective action upon the haemolymph. The amount of sodium injected was 8·5 μequiv., so that at a rate of excretion of 1·1 μequiv. per day it would take over a week for the haemolymph to return to normal. From the figures in Table 6 the average concentration of sodium in the urine is 22 m.equiv./l. Taking the average rate of flow as 3·5 mm.^{3}/hr. it can be calculated that sodium is excreted by the Malpighian tubules at a rate of 1·8 μequiv. per day. This means that

Table 5. *Changes in haemolymph during fasting*

Hr.	Insect no. 1			Insect no. 2			Insect no. 3			Insect no. 4			Insect no. 5			Insect no. 6		
	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.
0	12	13	161	11	14	154	10	15	159	10	17	153	10	14	167	11	14	163
48	14	12	159	13	13	156	11	10	160	11	13	160	13	14	160	13	14	160
96	12	11	160	10	12	154	10	12	167	11	12	161	10	17	151	12	13	154

Na and K in m.equiv./l.; O.P. in mM./l. NaCl.

Table 6. *Changes in haemolymph after injection, nos.* 1–3 *with* NaCl, *nos.* 4–6 *with* KCl

Hr.	Insect no. 1			Insect no. 2			Insect no. 3			Insect no. 4			Insect no. 5			Insect no. 6		
	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.	Na	K	O.P.
0	12	11	160	10	12	154	10	12	167	11	12	161	10	17	151	12	13	154
1	48	8	—	49	10	—	51	10	—	9	42	—	8	54	—	9	45	—
6	45	9	—	45	10	—	49	12	—	9	23	—	6	70	—	9	38	—
24	42	9	—	44	11	—	47	10	—	9	19	—	6	82	—	10	24	—
72	31	10	161	38	11	—	38	10	157	10	14	152	—	—	—	—	—	—
96	—	—	—	35	11	150	—	—	—	—	—	—	—	—	—	—	—	—
Urine	21	126	147	22	126	—	24	124	154	5	148	148	—	—	—	—	—	—
I.F.	47	107	159	35	99	162	42	99	157	3	149	180	—	—	—	—	—	—

Na and K in m.equiv./l.; O.P. in mM./l. NaCl. I.F. = intestinal fluid.

0·7 μequiv. per day must be reabsorbed at a time when the excretory system could best serve the insect by eliminating the maximum possible quantity of sodium.

In the case of potassium the concentration falls very much more rapidly (except in no. 5, in which the opposite change suggests general breakdown of the cell membranes). The return towards the normal concentration has progressed half way in about one day. Unfortunately these insects passed no faeces so it is not possible to estimate how much of this progress can be attributed to excretion. The evidence of the urine and intestinal fluid of the single insect examined indicate an increase in the rate of potassium excretion and a decrease in the rate of sodium excretion. It is also possible that some regulation occurs by potassium being taken up by the tissues, as was found by Tobias (1948) in the cockroach. It is certainly true that potassium can be given out by the tissues since it is possible to continue collection of urine for 6 hr. or more, and to recover from the urine much more potassium than could have been present originally in the haemolymph.

DISCUSSION

Existing knowledge of excretion in insects has been adequately reviewed (Wigglesworth, 1953; Roeder, 1953), and discussion will therefore be limited to such new facts about the stick insect as are here presented. When the scope of the investigation involves several organs and covers processes in which several stages can be recognized, it is scarcely possible to be both brief and comprehensive without being superficial. Investigations of this type, seeking to provide a general outline, tend to leave behind them more problems than they set out to solve. It will be necessary throughout this discussion to draw attention to many questions which have been left open.

The analyses of serum broadly confirm the figures given by other workers for the haemolymph. They extend existing knowledge only in respect of phosphate which is present in rather unexpectedly high concentration. As stated earlier both haemolymph and serum appear to be supersaturated with magnesium phosphate—that is to say, it is not possible to make up an artificial solution containing these ions at the same concentrations and at the same pH as in serum—but what this means in physico-chemical terms is not easy to see. The apparent anion deficit is possibly met by amino-acids of the acidic type. This is not incompatible with the view that most of the non-protein amino-acids are present as peptides. It is a little surprising not to find evidence of more than two free amino-acids in substantial concentration, and it may be added that preliminary chromatograms in which different samples of serum were tested revealed a certain lack of uniformity. It seems likely that the relationship between free amino-acids, peptides and proteins in the haemolymph is a labile one, depending no doubt on the state of nutrition as well as on other factors.

The analyses of urine, on the other hand, provide more complete information about the composition of the fluid produced by the Malpighian tubules than is available for any other insect. In insects it is not usually possible to collect this fluid without admixture of fluid from the midgut or without exposing it to the

action of the rectal glands, and most of the 'urines' which have been analysed are in fact the fluids collected from the anus. The urine as collected in the present work does not include any contribution from the Malpighian tubules of the third type ('appendices of the midgut'), but this probably does not amount to more than 10% of the total. That the urine contains much potassium and little sodium was already known (Ramsay, 1953*b*), and the reabsorption of these ions in the rectum was not unexpected since this is known to occur in the mosquito larva (Ramsay, 1953*a*). The relation between the concentrations in the haemolymph and in the urine of these two ions has been more fully studied in another paper (Ramsay, 1955). It would be interesting to have similar information about the other inorganic ions; in particular it would be interesting to compare the excretion of calcium in the adult and in the nymph.

Very little can be said about nitrogenous excretion beyond that uric acid is present in the haemolymph and is definitely concentrated in the urine. It can be calculated that about 0·06 mg. of uric acid per day is eliminated by the Malpighian tubules, much less than in the smaller insect *Rhodnius* which excretes about 0·5 mg. of uric acid per day (Wigglesworth, 1931*a*). The deep brown colour of extracts of faeces makes it impossible to apply colorimetric methods directly, but chromatography shows the presence of uric acid in the faeces and gives no definite indication of allantoin or urea in faeces or in urine.

The action of the excretory system is modified so as to restore the normal composition of the haemolymph after this has been disturbed by injection of sodium or potassium, but its ability to do so is not great. It has been shown that 8·5 μequiv. of sodium injected into the haemolymph is eliminated at a rate of 1·1 μequiv. per day, with prospect of restoring the normal composition of the haemolymph in about a week. When one considers that *Rhodnius* takes in about 13 μequiv. of sodium at a meal and gets rid of most of it in 3 hr. (calculated from Wigglesworth, 1931*a*) while maintaining the composition of its haemolymph remarkably constant (Ramsay, 1952), one cannot avoid the conclusion that the stick-insect's powers of regulation are relatively feeble.

SUMMARY

1. The excretory system (Malpighian tubules and hindgut) of the stick insect is described, in extension of the earlier description by de Sinéty.

2. Analyses have been made of the inorganic components of serum (haemolymph heat-coagulated and centrifuged), of urine (the fluid produced by the Malpighian tubules), of faeces, eggs and privet leaves.

3. In the adult female insect most of the calcium ingested leaves the body with the eggs.

4. The haemolymph has the characteristic mineral base pattern of the herbivorous insect; it appears to be supersaturated with magnesium phosphate. Its volume varies from 70 to 180 mm.^{3}, with an average value of 130 mm.^{3}, representing about 15% of the weight of the insect.

5. The urine has the usual high concentration of potassium; except for phosphate the other inorganic components are present in lower concentration than in the haemolymph. Urine is produced at a rate of about 6 mm.^{3}/hr., implying complete turnover of the water of the haemolymph every 24 hr.

6. Except for a slight (20%) decrease in potassium concentration the composition of the haemolymph remains constant during fasting.

7. The regulatory powers of the excretory system have been tested by injecting NaCl and KCl into the haemolymph and are shown to be relatively feeble.

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THE EXCRETION OF SODIUM, POTASSIUM AND WATER BY THE MALPIGHIAN TUBULES OF THE STICK INSECT, *DIXIPPUS MOROSUS* (ORTHOPTERA, PHASMIDAE)

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INTRODUCTION

Evidence has been published (Ramsay, 1953) that in a variety of insects potassium is actively secreted into the urine, in which it is present in very much higher concentration than in the haemolymph. In that paper the suggestion was made that the active secretion of potassium into the tubule might be an essential process in the formation of urine. 'It seems possible that the active secretion of potassium, accompanied by some anion, might produce a high osmotic pressure in the tubule which would cause water to pass inwards through the wall; and that this in its turn would promote a passive diffusion of sodium into the tubule.' If this simple hypothesis is true it follows that the urine can never be hypotonic to the haemolymph. This hypothesis was put to the test on the Malpighian tubules of the stick insect (Ramsay, 1954) and clearly shown to be untenable; the urine could be, and often was, slightly but definitely hypotonic to the haemolymph, showing that water as well as potassium could be actively secreted against a gradient. This disposes of the hypothesis in its simplest form, but does not negative the suggestion that active secretion of potassium is an essential process in the formation of urine. The experiments to be described in this paper were undertaken in the first place in order to establish the relation between the rate of urine formation and the concentrations of potassium in the urine and medium, and in the second place to study the movement of sodium in relation to active transport versus passive diffusion.

The design of such experiments is dominated by certain practical considerations. Wigglesworth (1931*a*) has commented upon the unsuitability of Ringer solution as a medium for the Malpighian tubules of *Rhodnius*, and up to the present the same applies in the case of the stick insect. The media used in experiments must therefore be based on haemolymph. The maximum volume of haemolymph which can be obtained from a single insect is about 0·05 ml. The life cycle is about 9 months. The fairly large culture maintained for this investigation yields about 1·5 ml. of haemolymph per month. Although it is a simple matter to make a preparation of the whole battery of Malpighian tubules as described in another paper (Ramsay, 1955) and thereby obtain urine in relatively large quantities, such a preparation requires about 0·5 ml. of medium to bathe it, and if the medium is largely haemolymph progress along these lines promises to be slow. For this reason it has been necessary to use single tubules isolated in drops of medium of about 50 mm.^{3} or less in volume.

MATERIAL AND METHODS

A description is given elsewhere (Ramsay, 1955) of the excretory system of the stick insect. The Malpighian tubules are of three kinds, of which only two, the superior and the inferior tubules, have been used in the present work. There is some slight gradation in the appearance of the superior tubules from one end to the other; the inferior tubules resemble the superior tubules over most of their length but end distally in a dilatation which is packed with white granules.

The fresh haemolymph of the stick insect is not a very convenient medium for physiological work since it almost invariably coagulates during the course of an experiment. It has been found that if the fresh haemolymph is heated to 100° C. for a few minutes and then centrifuged a clear fluid, which will be called 'serum', is separated from a compacted clot. As a physiological medium for Malpighian tubules serum does not appear to be in any way inferior to haemolymph (see later). An analysis of the main inorganic constituents of serum is described in the paper referred to above and is reproduced for convenience in Table 1, col. 1, of the present paper.

Table 1

	Serum (1)	Ringer (2)
Sodium	11 m.equiv./l.	17 m.equiv./l.
Potassium	18 m.equiv./l.	15 m.equiv./l.
Calcium	7 m.equiv./l.	6 m.equiv./l.
Magnesium	108 m.equiv./l.	132 m.equiv./l.
Chloride	87 m.equiv./l.	150 m.equiv./l.
Phosphate (as $PO_4^{\equiv}$)	39 m.equiv./l.	30 m.equiv./l.
Sucrose	0	100 mM./l.
Δ	171 mM./l. NaCl	172 mM./l. NaCl
pH	6·6	6·7

A Ringer solution approximating to serum in composition can be prepared in the following way: 20 ml. M-H_3PO_4, 20 ml. M-NaOH + M-KOH, 80 ml. M-$MgCl_2$ and 50 ml. 0·1 M-$CaCl_2$ are mixed, made up to 1000 ml. with distilled water and brought to pH 7·0. An extensive precipitate is formed; this is filtered off and the filtrate is brought to pH 6·7. Its osmotic pressure is roughly equivalent to 120 mM./l. NaCl. 34 g. sucrose are then added to raise the osmotic pressure to about 170 mM./l. NaCl. Analysis gives the figures of Table 1, col. 2. The Malpighian tubules do not survive well in this fluid unless serum is added to it, but it can be used as a dissecting fluid for preparation of the tubules.

An earlier paper (Ramsay, 1954) describes the method of setting up a preparation of an isolated Malpighian tubule in a droplet of medium under liquid paraffin. This type of preparation lends itself not only to the collection of urine but also to the measurement of the electrical potential difference (p.d.) across the wall of the tubule, according to the method used in earlier work (Ramsay, 1953). A positive sign indicates that the lumen of the tubule is positive with respect to the medium.

Osmotic pressure was measured by freezing-point depression (Ramsay, 1949) and expressed as that concentration of NaCl, in mM./l., having the same osmotic

pressure. The average of two readings was taken, and the standard error is approximately ± 1 mM./l. NaCl. Concentrations of sodium and potassium were determined by flame photometry (Ramsay, Brown & Falloon, 1953). It is difficult to describe the accuracy of this method concisely. The great imponderable in flame photometry is interference error, arising from the presence of substances other than sodium and potassium in the fluid under investigation. In comparisons between different fluids, such as haemolymph and urine, having different backgrounds of possibly interfering substances, serious errors may arise. This difficulty has been met as far as possible by making up the standard solutions for haemolymph, serum and Ringer to contain 100 m.equiv./l. of magnesium and 10 m.equiv./l. of calcium (and correspondingly for urine 30 m.equiv./l. of magnesium and 10 m.equiv./l. of calcium) and by swamping variations in phosphate with excess of ammonium phosphate. It would be reasonable to assume that for purposes of comparison between haemolymph and urine the figures are accurate to ± 10%. Where two samples of haemolymph (or two samples of urine) are compared the method is very much more reliable, and indeed where the observations on the two samples are made alternately in the same 'run' interference errors can be disregarded and differences in concentration can be assessed as significant or otherwise by appropriate statistical treatment of the observations.

Volumes of medium, of the order 1–50 mm.^{3}, were measured in capillary pipettes. Volumes of urine, generally less than 1 mm.^{3}, were obtained by measuring with an eye-piece micrometer the diameter of the droplet as it was allowed to sink through liquid paraffin. These measurements are probably accurate to ± 10%, which in view of the variations in the rate of urine production is adequate for present purposes.

All experiments were carried out at room temperature, 14–17° C.

RESULTS

(1) *The preparation and testing of artificial media.* It has been stated above that serum is a satisfactory medium for the physiological study of Malpighian tubules. The evidence for this is presented in Fig. 1, which summarizes the results of an experiment with a superior tubule immersed in serum under liquid paraffin. It is shown that the tubule can continue to secrete urine of normal composition for over 24 hr. The performance of tubules in fresh haemolymph is not noticeably different.

On the other hand, the inferiority of Ringer as compared with serum is striking as is shown by the figures of Table 2.

It is therefore obvious that conclusions drawn from experiments carried out in a medium of pure Ringer would be open to the objection that the tubules were in an abnormal state. Satisfactory conditions, however, can be produced by using Ringer to which some serum has been added. In a medium containing 3 parts Ringer to 1 part serum the tubules survive for more than 8 hr. and produce urine at a rate which is of the same order as when the medium is pure serum. This

provides a basis for the preparation of media enriched or deficient in sodium and potassium, as will be described in subsection (4).

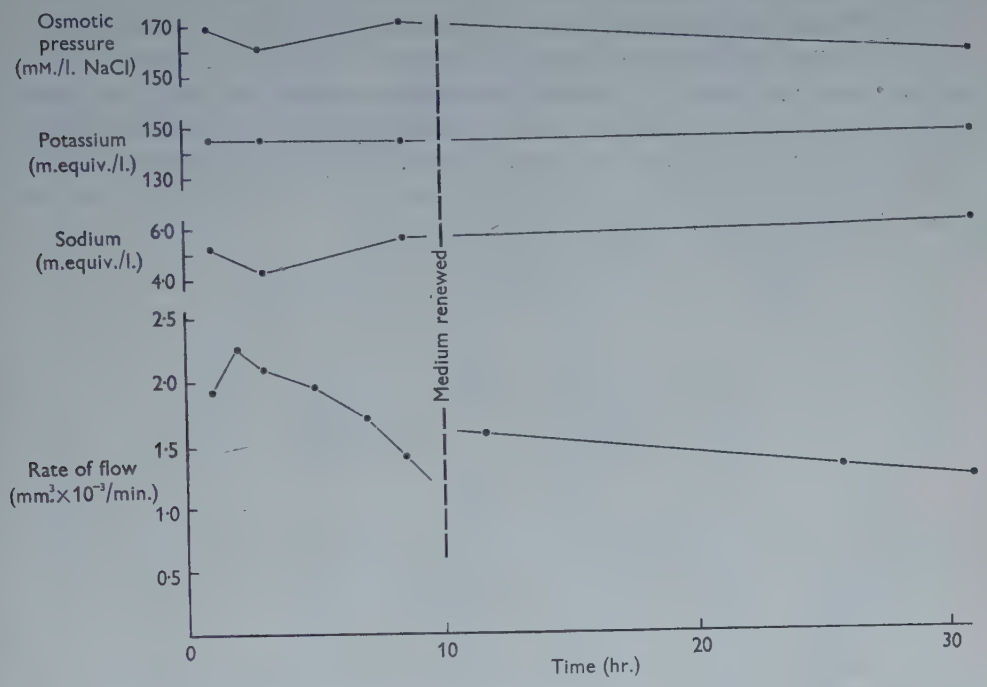

Fig. 1. Showing the ability of the tubule to maintain a flow of normal urine when bathed in serum. The points are plotted against the times at which the collections were made. Urine flow continued after 30 hr., further collections being made at 32 and 46 hr., but these were not analysed.

Table 2

Medium	Rate of urine flow during first 2 hr. (mm.^{3} × 10^{-3}/min.)	Duration of urine flow (hr.)
Pure serum	1·24	>24
Pure serum	1·59	>24
Pure Ringer	0·28	<9
Pure Ringer	0·65	<5
1 pt. serum, 3 pts. Ringer	1·33	>8
1 pt. serum, 3 pts. Ringer	2·28	>8

(2) *Regional differentiation in the Malpighian tubules.* In the superior tubule of the stick insect there is a slight gradation in appearance from one end to the other, and it was considered necessary to investigate the possible existence of a corresponding gradation in physiological properties.

The tubule was cut into three approximately equal lengths. Each length was then prepared for collection of urine under liquid paraffin as if it were a whole tubule, and all three lengths were immersed in the same droplet of serum, of

about 25 mm.^{3} volume. After a suitable interval the urine produced by each length of tubule was collected for volume measurement and analysis. The p.d. across the wall of the tubule was then measured over each of the three lengths.

The results of four such experiments are presented in Table 3. It is at once clear that there is some gradation of physiological activity in that the sodium/potassium ratio of the urine is greater at the proximal end than at the distal end, consistently so in all four cases. The rate of urine production, measured per unit length, is consistently greatest in the middle region. The p.d., which in all cases is such that the inside is positive to the outside, shows a tendency to be greatest in the distal region, but this tendency is not statistically significant.

Table 3

Tubule	Region	Sodium (m.equiv./l.) (1)	Potassium (m.equiv./l.) (2)	Osmotic pressure (mM./l. NaCl) (3)	Rate of flow ($mm.^3 \times 10^{-3}$/min.) (4)	Length (cm.) (5)	Rate of flow per unit length (6)	$p.d._{eq.}$ (Na) (7)	$p.d._{meas.}$ (8)	$p.d._{eq.} - p.d._{meas.}$ (9)
1	Proximal	9·9	102	165	0·17	0·4	0·44	+ 5	+33	−28
	Middle	6·0	128	167	0·70	0·7	1·00	+18	+31	−13
	Distal	2·9	150	172	0·67	0·7	0·97	+36	+33	+ 3
2	Proximal	10·1	95	162	0·24	0·5	0·48	+ 5	+12	− 7
	Middle	6·8	113	157	0·70	0·7	1·00	+15	+15	0
	Distal	3·9	138	165	0·33	0·7	0·47	+29	+24	+ 5
3	Proximal	8·9	112	163	0·46	0·95	0·48	+ 7	+35	−28
	Middle	8·3	135	163	0·67	0·8	0·84	+ 9	+38	−29
	Distal	5·2	155	171	0·56	0·7	0·80	+21	+36	−15
4	Proximal	10·0	75	180	0·25	0·8	0·31	+ 5	+25	−20
	Middle	6·8	140	161	0·72	0·8	0·90	+14	+34	−20
	Distal	4·4	145	176	0·15	0·9	0·17	+25	+33	− 8

A large number of collections of urine from both superior and inferior tubules have been analysed for sodium, potassium and osmotic pressure, and no significant differences between the two types of tubule have been noted. The proximal and middle regions of the inferior tubule are closely similar in appearance to the corresponding regions of the superior tubule. It is therefore probable that the same gradation in physiological properties is present in these regions of the inferior tubule. On the other hand, the appearance of the distal dilatation of the inferior tubule suggests that its properties may be quite different.

There does not appear to be any measurable passage of water across the wall of the distal dilatation. If the dilatation is cut off and its cut end drawn out from a droplet of serum into the surrounding liquid paraffin no urine is secreted; and conversely, if the dilatation is drawn out into the liquid paraffin while still in connexion with the rest of the tubule in the serum no fluid accumulates around it. But there is some transfer of potassium from the lumen to the medium, as has been demonstrated in the following way.

Two droplets of serum were placed under liquid paraffin about 1 mm. apart. The proximal end of the tubule was closed by a ligature and the tubule was arranged so as to bridge across the gap between the two droplets, the proximal and middle regions being in one droplet (about 25 mm.^{3}) and the distal dilatation in the other (generally < 1 mm.^{3}). The short length of tubule between the droplets was supported on a fine platinum hook. Samples were taken from the distal droplet at the beginning and end of the experiment, and, as Table 4 shows, an increase in potassium concentration was always detected.

Table 4

Initial concentration (m.equiv./l.)		Final concentration (m.equiv./l.)		Duration of exp. (min.)
Na	K	Na	K	
12·2	19·5	12·5	24·7	450
12·1	19·5	12·5	20·9	450
12·7	18·9	11·8	23·4	360
12·8	17·9	11·1	24·2	360

Table 5

	Initial concentration (m.equiv./l.)		Final concentration (m.equiv./l.)		Duration of exp. (min.)
	Na	K	Na	K	
Proximal droplet	20·2	17·8	21·4	7·4	200
Distal droplet	20·4	25·9	20·4	36·8	200

In one experiment where the two droplets were both small and of about the same size it was possible to demonstrate the transfer of potassium from one droplet to the other (Table 5).

Since there is no movement of fluid this transfer of potassium presumably occurs by diffusion along the lumen of the tubule, aided by convection produced by the writhing movements.

It seems likely that the amount of potassium passed back into the haemolymph via the distal dilatation is small compared with the amount of potassium which is passed into the gut with the urine. In this respect the experiments just described are open to the objection that the ligature applied to the proximal end, by preventing the escape of urine, undoubtedly leads to distension of the tubule which in its turn may facilitate the escape of potassium through the distal dilatation. An experiment was therefore carried out in which the proximal end was left open and the urine allowed to escape. The results of this experiment are presented in Table 6, from which it can be seen that the amount of potassium returned to the medium through the distal dilatation is insignificant compared with the amount leaving the proximal end as urine.

Notwithstanding the gradation in physiological properties revealed in the experiments described above, it was decided that the whole of the superior tubule

could be regarded as a single physiological unit for purposes of investigating the effects of variation in the composition of the medium. This decision is further examined in the Discussion.

Table 6

Tubule	Distal droplet					Urine		
	K initial (m.equiv./l.)	K final (m.equiv./l.)	K difference (m.equiv./l.)	Volume ($mm.^3 \times 10^{-3}$)	K transported ($\mu equiv. \times 10^{-3}$)	K (m.equiv./l.)	Volume ($mm.^3 \times 10^{-3}$)	K transported ($\mu equiv. \times 10^{-3}$)
1	19·4	22·4	3·0	257	0·77	139	310	43
2	19·2	20·2	1·0	148	0·15	129	310	40

(3) *Active transport of sodium.* In order to decide whether an ion is actively transported or whether its movements can be accounted for by passive diffusion it is necessary to know not only the concentration gradient but also the p.d. Following the procedure adopted in earlier work (Ramsay, 1953), $p.d._{eq.}$ is calculated from the sodium concentrations in the medium and in the urine according to the formula

$$p.d._{eq.} = -58 \log \frac{Na_{urine}}{Na_{medium}}.$$

The measured value of p.d., $p.d._{meas.}$, is then subtracted algebraically from $p.d._{eq.}$ and if the difference is negative then active transport of sodium must be assumed.

The relevant information is assembled in Table 3, cols. 7–9, from which it is seen that $p.d._{eq.} - p.d._{meas.}$ is negative in all except three cases. This shows that the tubule is capable of transporting sodium against an electrochemical gradient, a fact which was not established in the earlier investigation referred to above. It may also be noted here, in anticipation of what is to be described in the next subsection, that in certain circumstances the sodium concentration may be greater in the urine than in the medium (see Fig. 5*b*).

(4) *Rate of flow and composition of urine in relation to sodium and potassium concentrations in the medium.* For the experiments to be described in this subsection only superior tubules have been used unless otherwise stated. The single isolated tubule is treated as a functional unit, the slight gradation in physiological properties (subsection 2) being disregarded.

Variants of Ringer, enriched or deficient in sodium and potassium, were prepared, 1 part of serum being added to 3 parts of Ringer, with further addition of sucrose, if necessary, to bring the osmotic pressure to about 170 mM./l. NaCl. The 'very high' concentrations of sodium and potassium could not be reached without exceeding this value for osmotic pressure, and it was therefore necessary in these cases to reduce the magnesium concentration to 40 m.equiv./l. For this

reason the observations made in these media are not quite on a level with the others.

The media, prepared as described above, were analysed for sodium and potassium at the end of each experiment, and the concentrations in m.equiv./l. were found to be of the orders given in Table 7.

Table 7

	'Normal'	'Low Na'	'Low K'	'High Na'	'High K'	'Very high Na'	'Very high K'
Na (m.equiv./l.)	17	4	17	54	17	91	17
K (m.equiv./l.)	16	16	6	16	53	16	89

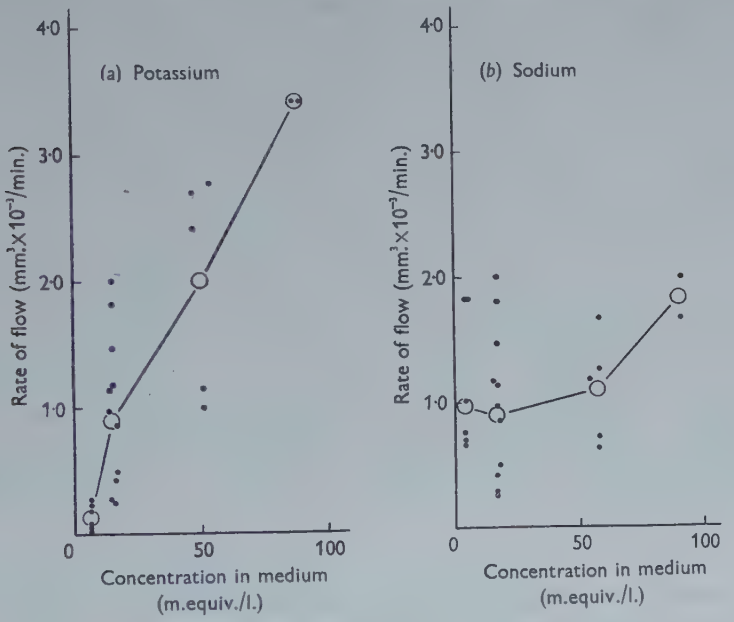

Fig. 2. (*a*) Rate of urine flow as a function of potassium concentration in the medium, the sodium concentration in the medium being constant at 16–17 m.equiv./l. (*b*) Rate of urine flow as a function of sodium concentration in the medium, the potassium concentration in the medium being constant at 15–16 m.equiv./l. The circles represent average values.

The rate of urine flow varies greatly from one insect to another, and the scatter of the observations makes it difficult to establish quantitative relations between the rate of urine flow and other factors. Nevertheless, it is very clear from Fig. 2*a* that with increase of the potassium concentration in the medium there is a very marked increase in the rate of flow, whereas with increase of the sodium concentration the increase in rate of flow is very much less and is barely significant in relation to the scatter of the observations (Fig. 2*b*).

The relation between potassium concentration in the medium and potassium concentration in the urine is shown in Fig. 3*a*, and the corresponding relation

from sodium concentrations in Fig. 3*b*. As expected from earlier work, the potassium concentration in the urine is always much greater than that in the medium, whereas the sodium concentration is generally less. Both curves show a tendency to rise steeply and then flatten off.

Further insight into these relations is gained by considering the rate of secretion of potassium (and of sodium) as a function of its concentration in the medium.

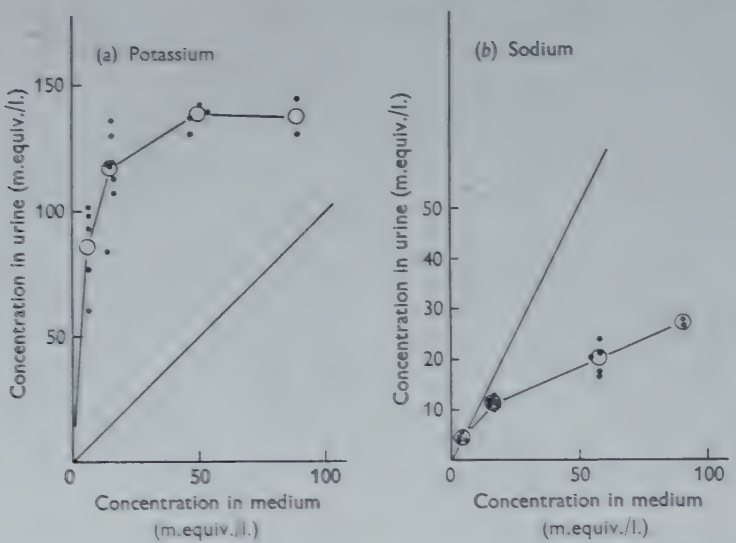

Fig. 3. (*a*) Potassium concentration in the urine as a function of potassium concentration in the medium, the sodium concentration in the medium being constant at 16–17 m.equiv./l. (*b*) Sodium concentration in the urine as a function of sodium concentration in the medium, the potassium concentration in the medium being constant at 15–16 m.equiv./l. The circles represent average values. The straight lines from the origin indicate equal concentration in urine and medium.

The average values for rate of flow (Fig. 2) are multiplied by the corresponding average values for concentration (Fig. 3) and the products are plotted in Fig. 4. From this it is seen that the rate of secretion of each ion can be regarded as roughly proportional to concentration of the ion in the medium, and that the rate of secretion of potassium is some 11 times greater than that of sodium.

Consideration has so far been restricted to the relation between the concentration of an ion in the urine and the concentration of the same ion in the medium. The possibility that the sodium concentration in the urine is affected by the potassium concentration in the medium (and vice versa) has still to be examined. As can be seen from Fig. 5*b* there is undoubtedly an inverse relation between the sodium concentration in the urine and the potassium concentration in the medium, and there is a suggestion of the same thing in the converse case (Fig. 5*a*). This at first sight suggests the possibility of mutual interference between sodium and potassium in the secretory mechanism. Allowance has to be made, however, for the increased flow of urine which accompanies an increase in the potassium concentration in the

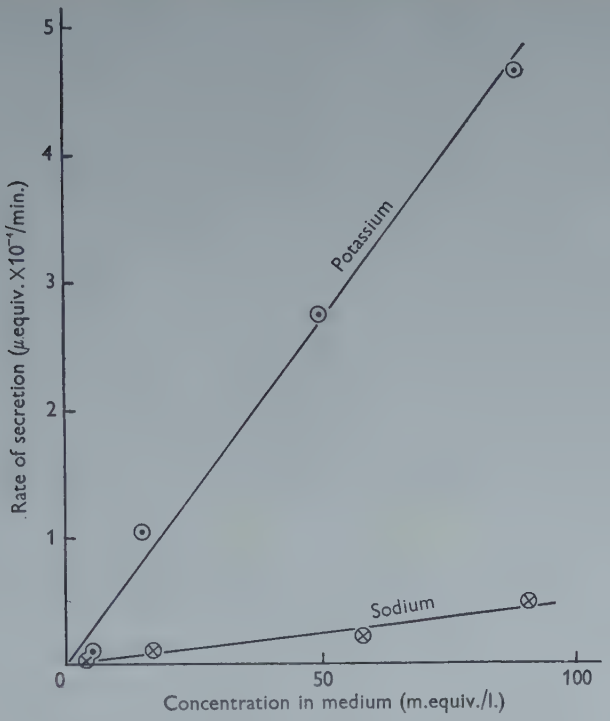

Fig. 4. The rate of secretion of potassium as a function of the potassium concentration in the medium (from data of Figs. 2*a* and 3*a*) and the rate of secretion of sodium as a function of the sodium concentration in the medium (from data of Figs. 2*b* and 3*b*).

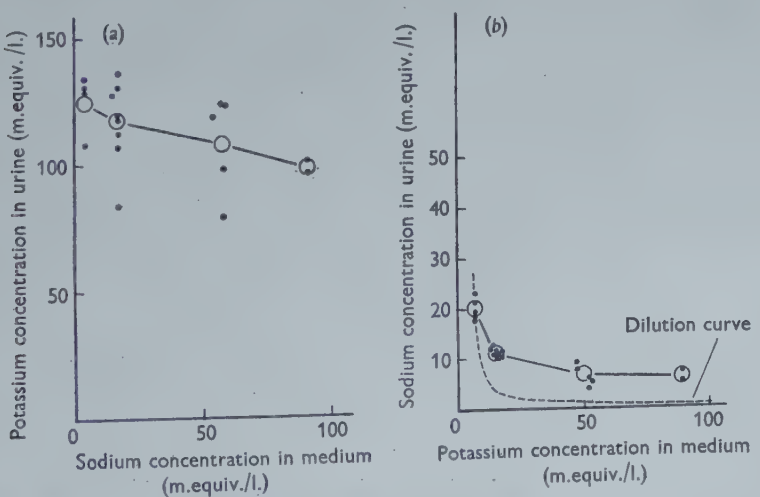

Fig. 5. (*a*) Potassium concentration in the urine as a function of sodium concentration in the medium, the potassium concentration in the medium being constant at 15–16 m.equiv./l. (*b*) Sodium concentration in the urine as a function of potassium concentration in the medium, the sodium concentration in the medium being constant at 16–17 m.equiv./l. The circles represent average values. For explanation of 'dilution curve' see text.

medium; even if sodium continues to be secreted at the same rate its concentration in the urine will fall as the rate of urine flow increases. From Fig. 5*b* the sodium concentration in the urine is 21 m.equiv./l. when the potassium concentration in the medium is 6 m.equiv./l. In the same medium, from Fig. 2*a*, the rate of urine flow is $0{\cdot}13 \times 10^{-3}$ mm.^{3}/min. The rate of secretion of sodium is therefore $2{\cdot}7 \times 10^{-6}$ μequiv./min. Assuming that the rate of secretion of sodium remains constant at this value and that the rate of urine flow varies with the potassium concentration in the medium as indicated in Fig. 2*a*, it is possible to calculate the expected sodium concentration in the urine for the other potassium concentrations in the medium. In this way the 'dilution curve' of Fig. 5*b* has been calculated and is seen to lie well below the observed values for the sodium concentration in the urine. This means that as the potassium concentration in the medium is increased and the rate of secretion of potassium increases the rate of secretion of sodium also increases, which does not suggest that sodium and potassium are in competition for the same secretory channel—at least, not under the conditions of these experiments.

The ability of the tubule to concentrate potassium is well marked. Fig. 3*a* shows that the ratio K_{urine}/K_{medium} increases as the potassium concentration in the medium is decreased, reaching a value of about 14 when the potassium concentration in the medium is 6 m.equiv./l. In view of this progressive increase and of the higher concentration ratios found for the Malpighian tubules of other insects, particularly those living in fresh water (Ramsay, 1953)* it seems possible that in the stick insect further decrease in the potassium concentration in the medium might reveal powers of concentration hitherto undisclosed. Unfortunately, by reason of the fact that it is necessary to add 1 part of serum to 3 parts of Ringer, artificial media having very low potassium concentrations cannot be prepared. There is, however, another means whereby the effect of very low potassium concentrations can be studied and that is by allowing the potassium concentration in the medium to be lowered by the activity of the tubule itself. The tubule can be immersed in a very small droplet of medium, and samples of urine and of medium can be taken at intervals. Since the potassium concentration in the medium is falling all the time it is more difficult to interpret the results of experiments carried out in this way, but at least they give some idea of the power of the tubule to remove potassium from low concentrations.

In one such experiment two tubules were set up in the same small droplet of serum. The results of the experiment are given in full in Table 8 and are plotted for one tubule only in Fig. 6. The concentration of potassium in the medium has been reduced to 0·4 m.equiv./l. and the corresponding concentrations in the urine are of the order of 50 m.equiv./l. When the concentration of potassium falls to 1 m.equiv./l. or less there is no doubt that the percentage error of analysis increases;

* In the paper to which reference is made there are two errors in Table 1. $\frac{C_1}{C_2}$ (Na) for *Dytiscus* should be 0·31 and $\frac{C_1}{C_2}$ (K) for the Tabanid should be 32·0.

Table 8

Time of collection (min.)	Medium					Urine, tubule (1)						Urine, tubule (2)					
	Sodium (m.equiv./l.)	Potassium (m.equiv./l.)	Osmotic pressure (mM./l. NaCl)	Volume of sample ($mm.^3 \times 10^{-3}$)	Potassium (μequiv. $\times 10^{-3}$)	Sodium (m.equiv./l.)	Potassium (m.equiv./l.)	Osmotic pressure (mM./l. NaCl.)	Volume ($mm.^3 \times 10^{-3}$)	Rate of flow ($mm. \times 10^{-3}$/min.)	Potassium (μequiv. $\times 10^{-3}$)	Sodium (m.equiv./l.)	Potassium (m.equiv./l.)	Osmotic pressure (mM./l. NaCl)	Volume ($mm.^3 \times 10^{-3}$)	Rate of flow ($mm.^3 \times 10^{-3}$/min.)	Potassium (μequiv. $\times 10^{-3}$)
0	11·5	17·3	177	—	—	—	—	—	—	—	—	—	101	—	235	1·62	23·7
145	12·0	10·0	178	84	0·84	6·5	125	165	210	1·45	26·2	—	85	153	165	1·32	14·0
270	11·2	5·9	171	100	0·59	7·3	113	164	190	1·52	21·6	7·8	79	157	260	1·11	20·5
505	12·3	3·6	171	155	0·56	8·6	93	165	280	1·19	26·1	8·5	71	155	180	0·95	12·8
695	11·7	0·5	165	103	0·05	13·5	76	165	155	0·81	11·8	11·7	51	147	148	0·23	7·5
1330	12·5	0·4	150	116	0·05	16·2	48	160	142	0·22	6·8	14·7	—	—	988	—	78·5
Totals	—	—	—	558	2·09	—	—	—	977	—	92·5	—	—	—			
Medium remaining at end of experiment		0·4		6200	2·60												

Original volume of medium: $6200 + 558 + 977 + 988 = 8723$

Amount of potassium originally present in medium: $8723 \times 17{\cdot}3 = 151\ \mu\text{equiv.} \times 10^{-3}$

Amount of potassium finally present in medium: 2·60

Amount of potassium in samples of medium: 2·09

$4{\cdot}69 \simeq 5{\cdot}0$

Amount of potassium lost from medium: $151 - 5 = 146\ \mu\text{equiv.} \times 10^{-3}$

Amount of potassium recovered in urine: $92{\cdot}5 + 78{\cdot}5 = 171\ \mu\text{equiv.} \times 10^{-3}$

Discrepancy: 14·6 %

but even making generous allowance for this it would seem that the ratio K_{urine}/K_{medium} can reach the order of 50.

The rise of the sodium concentration in the urine as the potassium concentration in the medium falls confirms the observations recorded in Fig. 5*b*.

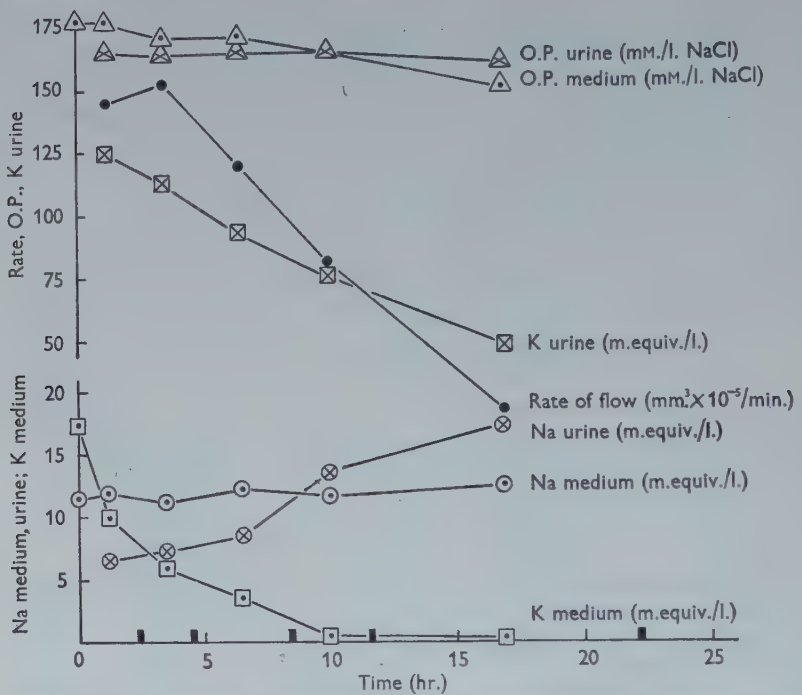

Fig. 6. Results of an experiment using a small volume of serum, showing the ability of the tubule to reduce the potassium concentration in the medium. The times of collection are indicated on the abscissa, and the points are plotted at the middle of the time intervals between collections.

Table 9

Tubule	Potassium lost from medium (μequiv. $\times 10^{-3}$)	Potassium recovered in urine (μequiv. $\times 10^{-3}$)	Discrepancy (%)
1	114	122	7
2	127	131	3

In experiments of this type it is possible to make out a balance sheet for potassium, comparing the amount of potassium which disappears from the medium with the amount which can be recovered from the urine. The object of preparing such a balance sheet is to provide a check upon the errors involved in measurements of concentration and of volume. In the case in question 146×10^{-3} μequiv. were lost from the medium and 171×10^{-3} μequiv. were recovered in the urine. This is a discrepancy of the order of 15%. In two other cases, in which inferior tubules were set up each in its own droplet of serum, the discrepancies were less (Table 9).

These figures give some assurance that the accuracy claimed for the methods used in this investigation has not been grossly overestimated.

DISCUSSION

The decision to treat the whole of the superior tubule as a single physiological unit may appear to take insufficient account of the very definite evidence for gradation in physiological properties.

In the Malpighian tubules of *Rhodnius* there is a striking discontinuity between the proximal and distal regions: urine is rapidly secreted in the distal region and appears to be partly reabsorbed in the proximal region (Wigglesworth, 1931*b*). Potassium is actively secreted in the distal region and is partly reabsorbed in the proximal region (Ramsay, 1952). In *Rhodnius* the composition of the urine as it leaves the tubule is therefore determined by the net effect of processes acting in opposition. If it were found that the potassium concentration in the urine increased with increasing potassium concentration in the medium this might be the result either of more rapid secretion in the distal region or less rapid reabsorption in the proximal region. It would therefore be necessary to study the two regions separately. But in the stick insect it has been found that sodium, potassium and water are secreted into the tubule at all levels. There is no indication that the opposing process of reabsorption is anywhere at work. The relative rates of secretion of sodium and potassium certainly do vary from one region to another; it may well be that some regions of the tubule respond more readily to changes in the medium than do others, and this possibility might be worth following up. It seems unlikely, however, that the general conclusions which have been reached from the investigation of the whole tubule will be contradicted by more detailed studies of its different regions.

While this position is maintained with respect to sodium, potassium and water, complete reservation must be made with respect to other constituents of the urine, which may be secreted in some regions and not secreted or even reabsorbed in others. In this connexion may be mentioned the observation that the urine produced by the proximal and middle regions is brown in colour, whereas that produced by the distal region of the superior tubule is colourless.

There is no evidence to suggest that the proximal and middle regions of the inferior tubule are in any way different from the corresponding regions of the superior tubule. The distal dilatation of the inferior tubule has quite different properties. It does not secrete urine and it is packed with granules in which calcium carbonate predominates (de Sinéty, 1901; Ramsay, 1955). Such evidence as there is suggests that it acts as a storage organ for calcium which is required in considerable quantity for hardening the shell of the egg (Moscona, 1950).

The main purpose of this investigation may be said to be achieved in the results presented in Figs. 2 and 3. Increase of the potassium concentration in the medium results in a spectacular increase in the rate of urine flow and also in an increase of the potassium concentration in the urine. On the other hand, increase of the sodium concentration in the medium results in a smaller increase of the sodium concentration in the urine and in a barely significant increase in the rate of urine flow. For both sodium and potassium the rate of secretion appears to bear a linear relationship to the concentration in the medium (Fig. 4); the simplest interpretation is that under

the conditions of these experiments the rate of secretion is limited by the availability of the ion and not by the failure of the secretory mechanism to transport all the ion that can be fed into it. But although the points in Fig. 4 lie reasonably near the lines it must not be forgotten that they represent average values to which considerable errors attach, so that the linearity of the relationship may be more apparent than real. Fig. 4 also serves to show the very great difference in the rates of secretion of sodium and potassium, and about this there is no doubt at all.

In the earlier investigation (Ramsay, 1953), which included the Malpighian tubules of the stick insect, it was stated that 'there are insufficient grounds for assuming the active transport of sodium, in fact, it is admissible as a working hypothesis that the differences in concentration of sodium are brought about by passive diffusion'. It is now possible to be more precise. In 'high Na' media the electrochemical gradient is such as to promote the diffusion of sodium into the tubule, but in 'normal' media and *a fortiori* in 'low K' media (where the sodium concentration in the urine can exceed that in the medium) active transport against the electrochemical gradient must take place. It is perhaps worth while to make clear the very limited implications of this statement. There is no suggestion that a different mechanism is involved according to whether the medium is of the 'low K' type (active transport) or the 'high Na' type (passive diffusion) any more than that a different mechanism is involved when a motor car is driven uphill under its own power or is allowed to run downhill with the engine being turned passively; in the one case energy must be supplied, in the other case energy need not be supplied, and that is all. It has now been shown that there must be a mechanism capable of transporting sodium against an electrochemical gradient, just as it has previously been shown that such a mechanism must exist for potassium. Although no evidence has been put forward to show that sodium and potassium are in competition for the same mechanism, the possibility that the same mechanism serves both ions is not excluded.

A great many measurements of p.d. were made upon tubules in various artificial media, but it does not seem worth while to report these in detail. It can be stated briefly that the p.d. is notably unresponsive to changes in the sodium and potassium concentrations in the medium. An average increase of 16 mV. was noted in 'high K' as compared with 'low K', that is, for an eightfold increase in potassium concentration; corresponding changes in sodium concentration were without effect. This speaks against the idea that passive diffusion plays an important part in the movements of sodium ion.

The results here presented reinforce the suggestion made in earlier papers that there is some connexion between the secretion of potassium and the processes of formation of urine. It has now been shown that the greater the concentration of potassium in the medium the greater is the flow of urine. At the same time, in so far as it has been demonstrated that sodium can be actively transported and seems unlikely to enter the tubule by passive diffusion as at one time seemed possible, potassium is no longer to be regarded as having a unique role. Some re-assessment of the position is called for.

There is no doubt that the Malpighian tubules of insects are generally very active in removing foreign materials (e.g. dyes) from the haemolymph. They are also active in removing the natural excretory products (e.g. uric acid). If the tubule merely removed these substances from the haemolymph to its lumen and allowed them to accumulate there, there would be a limit to its usefulness; it is obviously advantageous from the insect's point of view that the excretory substances should be rapidly flushed out of the tubule and so eliminated from the body via the hindgut. This might be brought about by the simple secretion of water into the tubule, but it appears that the tubule is not able to secrete water against anything more than a slight osmotic gradient. It follows that a brisk flow of urine can only be maintained by the secretion of other substances into the urine to make up the osmotic difference. Potassium ion appears to be one of the most important of these substances. Just as most of the water secreted by the tubules is reabsorbed in the hindgut, so also is most of the potassium reabsorbed (Ramsay, 1955). The circulation of potassium is thus bound up with the circulation of water and its significance is to be traced to the inability of the tubule to secrete water rapidly against a concentration gradient.

This interpretation, however, entirely begs the question of why it is mainly potassium rather than sodium which is circulated. The tubule is capable of actively secreting sodium, at least in the stick insect. In many insects sodium is available in the haemolymph in much higher concentration than potassium. The fact remains that in the stick insect, and possibly in many other insects, the mechanism of potassium secretion works very much faster than the mechanism of sodium secretion. Why this should be so is at present a matter of pure speculation.

Of the many problems associated with the study of urine formation in Malpighian tubules none is more challenging than the problem of why the secretory cells are unable to function in balanced salt solutions similar in composition to the haemolymph. It is striking to observe how the contractile elements of the tubule wall can maintain normal activity in Ringer solution long after the secretory cells have completely disintegrated. All the evidence suggests that it is not a question of high sensitivity to the precise composition of the Ringer, but that it is a question of the absence of some substance which is present in the natural medium. Attempts have been made, using the methods of partition chromatography, to separate this essential principle from serum, but so far it has not been possible to recover from the paper any substance which when added to Ringer will significantly prolong the effective life of the secretory cells. Further investigation of this problem will be undertaken when adequate supplies of serum have been accumulated.

SUMMARY

1. The excretion of sodium, potassium and water by the Malpighian tubules of the stick insect has been further studied in preparations of single tubules isolated in droplets of medium under liquid paraffin.

2. There is some gradation of physiological activity along the length of the superior tubule. Sodium, potassium and water are secreted into the tubule at all levels, but the sodium/potassium ratio is greater in the proximal region.

3. The proximal and middle regions of the inferior tubule have not been shown to differ in any way from the corresponding regions of the superior tubule. The distal dilatation has quite different properties and does not produce urine.

4. The rate of urine flow increases markedly as the potassium concentration in the medium is increased; the corresponding effect of sodium is barely detectable.

5. Sodium, like potassium, can be actively transported against an electrochemical gradient, and does not appear to compete with potassium in the secretory mechanism.

6. The rates of secretion of sodium and potassium vary in direct proportion to the respective concentrations of these ions in the medium. The rate of secretion of potassium is more than ten times greater than that of sodium.

I wish to thank Prof. A. L. Hodgkin for reading the manuscript of this paper.

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